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TITLE: Population Based Assessment of MHC Class I Antigens Down Regulation as Markers of Increased Risk for Development and Progression of Breast Cancer from Benign Breast Lesions

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13. ABSTRACT (Maximum 200 Words)
Despite advances in chemotherapy and radiation therapies, advanced breast cancer still carries a high mortality rate. The need for effective therapies is urgent. The overall aim of this research proposal is to recognize early markers of disease and their interaction with other epidemiological risk factors that can serve as risk indicators for subsequent development of breast cancer from precancerous lesions, and as prognostic markers for progression from primary to metastatic disease. The major histocompatibility complex (MHC) class I molecules are found on the cell membrane of all cells in the body and are involved in intercellular communications and in complex interactions with the immune system. Cancer cells with reduced or aberrant MHC molecules have been shown to evade immune surveillance and become selected for cancer progression and spread of disease to distant sites of the body. About half of all breast cancers have complete loss of reduced level of MHC class I molecules and this finding has been associated with increased tumor invasiveness and more aggressive cancers with poorer outcome. The outlined studies are expected to better define the clinical significance of abnormal MHC class I molecules in precancerous and invasive breast lesions as markers of immunological events that could affect survival, selection, and outgrowth of precancerous cells, and their subsequent progression to breast cancer. These MHC losses could also mark more aggressive tumors and thus contribute to selection of appropriate treatments in individual cases.

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Major histocompatibility complex (MHC) class I molecules, disease progression markers, survival, breast cancer

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INTRODUCTION

It has been known for some time that malignant transformation of cells is frequently associated with abnormalities in the expression of MHC class I antigens (1). These abnormalities appear to play a role in the clinical course of the disease (1) and to have a negative effect on the outcome of T cell-based immunotherapy for malignant diseases (2, 3). In breast lesions examined for expression of MHC class I, approximately half (51%) of carcinomas had an abnormally low content of HLA-A, -B, and -C determinants (4). Down regulation of HLA class I antigens in breast carcinomas may be more frequent than previously reported suggesting that alterations of HLA class I could represent an important step associated with tumor invasion providing tumor cells with the ability to escape recognition by T-lymphocytes (5). The overall aim of our research is to better define the role of MHC class I antigen loss and its interaction with other histo-pathologic and epidemiological factors that can serve as risk indicators in the progression of primary breast cancer to metastatic disease.

BODY: Statement of Work

TASK 1:

In women with primary and metastatic lesions of the breast to determine whether HLA Class I antigen loss and down regulation is greater in those with late stage and metastatic disease than in women with early stage disease (months 1-48); to determine whether among women with concurrent preneoplastic lesions and breast tumors HLA Class I antigen loss or down regulation is more frequent in the tumor than in the pre-neoplastic lesion (1-54); association with histopathologic characteristics of the lesions, including estrogen and progesterone receptor status (months 1-54); and disease survival (1-58).

a: Begin construction of the breast cancer cohort (3000 cases). The Pathologist Dr.Raju and the P.I will begin screening breast cancer cases for delineation into Stage1-IV, and for the presence of concurrent lesions of benign proliferative and cancer lesions, together with normal breast tissue. We will design appropriate forms to record histopatholgical and clinical data based on our current NIH project forms and instruments (Instruments section in original grant)

b: Retrieval of H & E slides for cases

c: Review of slides

d: selection of tumor blocks and sectioning of tissue for immunohistochemistry assays

e: begin HLA class I immunoassays, as slides become available

f: Continue construction of the breast cancer cohort, the concurrent lesion cohort and the histopathologic data gathering. See Pathology Review Form (PRF) (Instruments Section) for histopathologic parameters.

g: continue HLA class I immunoassays as additional cases are entered into the cohort

h: Annual reports will be written

i: Initial manuscripts on the PBD cohort will be written

PROGRESS (January 1, 2003- December 31, 2003):

1. The finalized Pathology Review Form (PRF) has been converted into the Teleform version for electronic data entry. Electronic data entry has been accomplished for 203 cases. We expect this number to increase substantially during 2004.
2. Thus far over 5139 pathology reports have been obtained. A finalized data base of HFHS breast cancer tumor Registry patients has been completed totaling 6338 cases.
3. H& E slides have been retrieved from the pathology archives for an additional 340 cases, for a total of 1290 cases.
4. 300 additional cases have been reviewed by the pathologist on PRF forms for a total of 1050 cases
5. Selection of tumor blocks completed for an additional 200 cases bringing the total cases to 780 thus far.
6. Sectioning of tissue for immunohistochemistry assays: completed for 700 cases.
7. HLA class I immunoassays: completed for 310 cases
8. The finalized HLA Immunohistochemistry Form has been converted into the Teleform version for electronic data entry Appendix #1).
9. Medical abstraction form: completed in 453 cases

TASK 2
Final analysis and report writing (months 56-60)

a: Final analysis of epidemiological risk factor data, histopathological and clinical data and HLA expression results will be performed.

b: A final report and additional manuscripts on the breast cancer cohort will be prepared

Progress: PENDING

KEY RESEARCH ACCOMPLISHMENTS

- The Henry Ford Health System Tumor Registry data, which is the source of our study cohort starting from 1981 through 2000 was validated and verified for vital patient information against the SEER data (2003 progress item). We have so far acquired a total patient database of 4,900 validated and verified breast cancer cases. The verification and validation of our constructed breast cancer cohort, completed as part of our 2003 progress goal has been followed up with a complete database construction of all pathology report numbers (surgical path numbers) numbering 5139. This a critical task as the HFHS Tumor Registry does not indicate the pathology report numbers. The latter is imperative for any tissue analysis which requires information of the path number and the specific tissue block. A major accomplishment has been the careful retrieval, and data entry of pathology reports for an updated database of 6338 patients.

- Manuscript Publications/Submissions:
  See Reportable Outcomes below.
- Completed a total of 310 cases for HLA assessment

REPORTABLE OUTCOMES

1. MANUSCRIPTS/REPORTS

A: DOWNREGULATION OF HLA-A AND BW6, BUT NOT BW4, ALLOSPECIFICITIES IN LEUKEMIC CELLS. AN ESCAPE MECHANISM FROM CTL AND NK ATTACK?
 Blood First Edition Paper, prepublished online December 4, 2003; DOI 10.1182/blood-2003-07-2500 (Appendix # 2)

B: HLA ANTIGEN EXPRESSION IN BREAST CANCER: A MULTICENTRIC STUDY UTILIZING FORMALIN-FIXED PARAFFINIZED TISSUES. M J. Worsham[1], R. Nanavati[1], U. Raju[1], S.R. Wolman[2], T. Cabrera [3], F. Garrido [3], E. A. Repasky[4], B. Hylander[4], M. Feenstra [5], M.Verdaasdonk[5], M.Schipper[5], M.Tilanus[5], S.Ferrone[4]. [1] Cancer Genetics Research, Department of Pathology, Henry Ford Health Systems, Detroit, MI, 48202, USA [2] Uniformed Services Univ., of the Health Sciences, Bethesda, MD 20814, USA, Hosp., Univ., Virgen de las Nieves, Granada, Spain, [4] Roswell Park Cancer Institute, Buffalo, NY 14263, [5] Univ., Hosp., Utrecht, The Netherlands.
Submitted to: *Breast Cancer Research* (Appendix #3)

C: SUBMITTED REPORT TO THE "HLA AND CANCER" COMPONENT OF 13TH INTERNATIONAL HISTOCOMPATIBILITY WORKSHOP, 8/24/03: LOSS OF HETEROZYGOSITY AT HLA LOCI 6P21 AND 15Q21 (Appendix # 4)

CONCLUSIONS:

A: DOWNREGULATION OF HLA-A AND BW6, BUT NOT BW4, ALLOSPECIFICITIES IN LEUKEMIC CELLS. AN ESCAPE MECHANISM FROM CTL AND NK ATTACK? (Appendix # 2)

HLA class I antigen defects may have a negative impact on the growing application of T cell based immunotherapeutic strategies for treatment of leukemia. Therefore in the present study taking advantage of a large panel of HLA class I allele-specific human monoclonal antibodies we have compared HLA class I antigen expression on leukemic cells with that on autologous and allogeneic normal cells. Downregulation of HLA-A and/or -B allospecificities was present in the majority of the patients studied. However, downregulation did not affect all HLA class I alleles uniformly, but was almost exclusively restricted to HLA-A allospecificities and to HLA-B allospecificities which belong to the HLA-Bw6 group. The latter allospecificities, at variance from those which belong to the HLA-Bw4 group, do not modulate the interactions of leukemic cells with NK cells. Therefore our results suggest that the selective downregulation of HLA-A and HLA-Bw6 allospecificities associated with HLA-Bw4 preservation provides leukemic cells with an escape mechanism not only from CTL, but also from NK cells. As a result T cell-based immunotherapeutic strategies for leukemia should utilize HLA-Bw4 alloantigens as restricting elements since a selective HLA-Bw4 allele loss would provide leukemic cells with an escape mechanism

B: HLA ANTIGEN EXPRESSION IN BREAST CANCER: A MULTICENTER STUDY UTILIZING FORMALIN-FIXED PARAFFINIZED TISSUES (Appendix # 3).

Despite the possible clinical significance and potential for T-cell based immunotherapy, evaluation of malignant lesions for HLA class I antigen expression is not performed routinely, even for patients who are candidates for such therapy. This reflects, at least in part, reluctance by pathologists to utilize frozen tissue sections in IHC assays. Little information is available about the usefulness of formalin-fixed paraffin-embedded tissues (FFPT) as substrates in IHC assays to evaluate tissue expression of HLA antigens. We therefore undertook a multicenter study to develop and standardize an IHC protocol using FFPTs and anti-HLA mAbs. To determine if loss of expression of MHC Class I molecules at the protein level reflect alterations at the gene level, DNA from microdissected normal and tumor tissue were evaluated with microsatellites at the MHC class I 6p21.3 locus (HLA-A, B, C determinants) and at the 15q21 beta 2 microglobulin locus for concordance of expression. HLA class I antigen down-regulation in conjunction with cellular heterogeneity of expression in three breast carcinoma cases was concordantly reported by the four participating laboratories with the anti-HLA class I antibody HC-10 and with the anti-beta 2 microglobulin L368. Furthermore, no staining of normal and malignant mammary cells was detected by the four laboratories in the lesions stained with the anti-HLA class II LGII. In contrast, infiltrating lymphocytes were strongly stained by LGII. Downregulation of class was reflected by LOH in cases 1 and 3 for the 15q21 locus and in case 1 at the 6p21 locus. The results indicate that FFPTs represent a useful substrate upon which to monitor HLA antigen expression in malignant lesions, especially when appropriate markers are used to differentiate malignant cells from lymphocytes and dendritic cells.

The manuscript has been submitted to "Breast *Cancer Research*" (Appendix #3).

C: **LOSS OF HETEROZYGOSITY AT HLA LOCI 6P21 AND 15Q21** (Appendix # 4)

To determine if loss of expression of MHC Class I molecules at the protein level reflect alterations at the gene level, the HLA and Cancer component of the 13th International Histocompatibility Workshop undertook a small pilot multicenter study. The goal was to optimize and assess the reproducibility of the assays to measure the expression of HLA class I antigens and to detect LOH at the HLA locus (6p21) and at the β2m locus in formalin fixed, paraffin embedded normal breast epithelium and malignant breast lesions.

DNA from microdissected normal and tumor tissue were evaluated with microsatellites at the MHC class I 6p21.3 locus (HLA-A, B, C determinants) and at the 15q21 beta 2 microglobulin locus for concordance of expression (Table 1). HLA class I antigen down-regulation in conjunction with cellular heterogeneity of expression in three breast carcinoma cases was concordantly reported by the four participating laboratories with the anti-HLA class I antibody HC-10 and with the anti-beta 2 microglobulin L368. Furthermore, the four laboratories detected no staining of normal or malignant breast tissue for the lesions stained with the anti-HLA class II LGII. In contrast, infiltrating lymphocytes were strongly stained by LGII. Downregulation of class I was reflected by LOH in cases 1 and 3 for the 15q21 locus and in case 1 at the 6p21 locus. The results indicate that FFPTs represent a useful substrate to monitor HLA antigen expression in malignant lesions, especially when appropriate markers are used to differentiate malignant cells from lymphocytes and dendritic cells.

Loss of heterozygosity for markers representative of the HLA loci at 6p21 and 15q21 agreed with down regulation of expression of class 1 gene expression. LOH associated with loss of the HLA locus at chromosome 6p supports an extended mechanism that may contribute to HLA haplotype loss previously described in different histological tumor types. Contaminating stroma can mask LOH results and

microscopic or laser microdissection, to separate stroma and tumor would facilitate an interpretation of LOH in cases with unclear results.

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APPENDIX

1: Study Instruments
HLA Immunohistochemistry Electronic Data Teleform

2: Manuscripts Published:
Downregulation of HLA-A and Bw6, but not Bw4, allospecificities in leukemic cells. An escape mechanism from CTL and NK attack? Copyright (c) 2003 American Society of Hematology. *Blood* First Edition Paper, prepublished online December 4, 2003; DOI 10.1182/blood-2003-07-2500

3: Manuscripts Submitted:
HLA antigen expression in breast cancer: a multicenter study utilizing formalin-fixed paraffinized tissues, submitted "Breast *Cancer Research*" (Appendix #3)

4: Report to 13th IHWG
Loss of heterozygosity at HLA loci 6p21 and 15q21, submitted 8/24/03

8283238251

DOD1 IHC FOR HLA

MRN ☐☐☐☐☐☐☐

Pathology_No ☐☐☐☐☐☐☐

*Block No (Digit) ☐☐

*Block No (Mix With Text and Digit) ☐☐☐

*Please fill only one of the two block Nos.

** L = light infiltration
M = moderate infiltration
H = heavy infiltration

Lesion	HC10	LG2	CD1A	CD57
☐ lymphocyte ** ☐L ☐M ☐H	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%
☐ Normal	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%
☐ N_Other	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%
☐ LN-N	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%
☐ LN-T	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%
☐ Tumor	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%
☐ DCIS	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%
☐ LCIS	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%
☐ BBD	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%	☐ 0% ☐ <10% ☐ 10%-25% ☐ 25%-50% ☐ >50%

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Article title: Downregulation of HLA-A and Bw6, but not Bw4, allospecificities in leukemic cells. An escape mechanism from CTL and NK attack?

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Running title: leukemic cells escape from CTL and NK cells

Abbreviations: ALL: acute lymphoblastic leukemia, AML: acute myeloid leukemia, CLL: chronic lymphoblastic leukemia, HLA: human leukocyte antigen, NK: Natural Killer cell, CDC: complement dependent cytotoxicity, MFI: mean fluorescence index.

Keywords: HLA class I, leukemia, downregulation, Bw4, Bw6, Natural Killer cell, immunosurveillance

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Abstract

HLA class I antigen defects may have a negative impact on the growing application of T cell-based immunotherapeutic strategies for treatment of leukemia. Therefore in the present study taking advantage of a large panel of HLA class I allele-specific human monoclonal antibodies we have compared HLA class I antigen expression on leukemic cells with that on autologous and allogeneic normal cells. Downregulation of HLA-A and/or -B allospecificities was present in the majority of the patients studied. However, downregulation did not affect all HLA class I alleles uniformly, but was almost exclusively restricted to HLA-A allospecificities and to HLA-B allospecificities which belong to the HLA-Bw6 group. The latter allospecificities, at variance from those which belong to the HLA-Bw4 group, do not modulate the interactions of leukemic cells with NK cells. Therefore our results suggest that the selective downregulation of HLA-A and HLA-Bw6 allospecificities associated with HLA-Bw4 preservation provides leukemic cells with an escape mechanism not only from CTL, but also from NK cells. As a result T cell-based immunotherapeutic strategies for leukemia should utilize HLA-Bw4 alloantigens as restricting elements since a selective HLA-Bw4 allele loss would provide leukemic cells with an escape mechanism from CTL, but would increase their susceptibility to NK cell-mediated lysis.

Introduction

The classical HLA class I molecules (HLA-A, -B, and -C) are expressed on the surface of most nucleated cells as a trimolecular complex composed of a 45 kDa polymorphic heavy chain (α-chain), a 12 kDa monomorphic light chain (β2-microglobulin) and a 9-11 amino acid long peptide. HLA class I antigens play an important role in the interaction of virus-infected and malignant cells with effector cells. They mediate recognition of target cells by cytotoxic T lymphocytes by presenting viral and tumor antigen derived 8-11 amino acid long peptides to the T cell receptor.[1] Furthermore HLA class I antigens modulate activity of NK cells by interacting with inhibitory and activating killer immunoglobulin-like receptors expressed on NK Cells.[2]

Analysis of a large number of cell lines and of surgically removed lesions has convincingly shown that defects in HLA class I antigen expression and/or function occur in almost every type of solid tumor, although the frequency of these abnormalities varies markedly among the various types of tumors (for review, see [3,4]). Defects in HLA class I antigen expression have been known for some time to have clinical significance, since they are associated with the clinical course of the disease in many types of malignant diseases (for review, see [4]). Nevertheless HLA class I defects in tumor cells have been of limited interest among tumor immunologists for many years. However, in recent years the interest in the characterization of HLA class I antigen expression in malignant lesions and of the molecular mechanisms underlying HLA class I defects has been rekindled by the potential negative impact of HLA class I abnormalities on the outcome of T cell-based immunotherapy. The latter is being applied with increasing frequency for the treatment of solid tumors thanks to the availability of well-defined tumor antigens to be used as immunogens in clinical trials.[5,6]

During the last few years numerous leukemia-specific translocations or overexpressed genes have been described both in myeloid and lymphoid leukemia cells.[7] The resulting gene products may eventually be utilized to implement T cell-based immunotherapy of leukemia, especially since a spontaneous or induced immune response to some of these moieties has

already been described.[8,9] These possibilities have stimulated interest in the characterization of HLA class I antigen expression by leukemic cells. Contrary to solid tumors, HLA class I antigen expression has been investigated only to a limited extent in hematological malignancies such as in B cell and Hodgkin lymphomas,[10,11] CLL,[12] ALL and AML.[13-15] Defects in HLA class antigen expression have been found only in a low percentage of the samples analyzed. Total HLA class I antigen loss has been associated with an aggressive clinical course of the disease in non-Hodgkin lymphoma[16] and with an increased expansion of the leukemic clone at diagnosis in AML.[17]

The scanty information about HLA class I antigen expression has a negative impact on the selection and design of immunotherapeutic strategies for the treatment of leukemia. Therefore in the present study taking advantage of a large panel of HLA class I allele-specific human monoclonal antibodies (mAb) we have compared the level of HLA class I antigen expression on acute and chronic leukemic cells with that on autologous and allogeneic normal lymphocytes.

Materials and methods

Cell lines

Cultured human lymphoid cells: K562 (HLA-A11, -A31, -B18, -B40), KARPAS-299 (HLA-A03, -A11, -B07, -B35), ML-2 (HLA-A02, -B44, -B51), MONO-MAC-6 (HLA-A03, -BO7, -B51), NALM-6 (HLA-A01, -A02, -B08, -B15), NB-4 (HLA-A11, -B35, -B40), RPMI-8402 (HLA-A01, -A29, -B07, -B38), and 697 (HLA-A02, -A25, -B07, -B15) were maintained in RPMI 1640 medium supplemented with 5% fetal calf serum, antibiotics and L-glutamine at 37°C in a humidified 5% CO_2 incubator.

Monoclonal and polyclonal antibodies

Anti-CD2 (clone leu 5b), anti-CD3 (clone leu 4), anti-CD5 (clone leu1), anti-CD16 (clone leu 11), anti-CD19 (clone leu 12), anti-CD45 (clone leucocyte) and anti-CD56 (clone leu19) mAb

were purchased from Becton Dickinson (Erembodegem, Belgium). Anti-CD13 (clone My7) and anti-CD33 (clone My9) mAb were purchased from Analis (Namur, Belgium).

The mouse mAb W6/32[18] which recognizes a conformational determinant expressed on β_2m associated HLA-A, B and C heavy chains, the mouse mAb LGIII-147.4.1[19] which recognizes a conformational determinant expressed on all β_2m associated HLA-A heavy chains except HLA-A9, the mouse mAb B1.23.2[20] which recognizes a conformational determinant expressed on all β_2m associated HLA-B and C heavy chains and the mouse anti-β_2m mAb NAMB-1[21] were developed and characterized as described. The mouse anti-HLA-DR (clone HLA-DR) and anti-HLA-DR, DQ, DP (clone Tu-39) mAb were purchased from Becton Dickinson.

Human mAb (hu-mAb) are secreted by Epstein Barr Virus transformed B-lymphocytes isolated from multiparous women who had developed HLA antigen-specific antibodies during pregnancy. HLA antibody producing EBV cell lines were stabilized by electrofusion and rigorous cloning.[22-25] The HLA specificity of mAb was determined by testing with a large (n>240) panel of HLA-typed peripheral blood mononuclear cell suspensions in the conventional complement dependent microcytotoxicity (CDC) assay. For flow cytometry application, HLA-Hu-mAbs were used as undiluted hybridoma culture supernatants.

Fluorescein isothiocyanate (FITC) conjugated $F(ab^1)_2$ fragments of goat anti-mouse IgG antibodies and FITC conjugated $F(ab^1)_2$ fragments of rabbit anti-human Ig antibodies were purchased from Prosan (Merelbeke, Belgium).

Leukemia cells

EDTA anticoagulated blood or bone marrow samples were obtained from 64 leukemia patients at diagnosis. The only selection criterion was CD19+ and CD33+ membrane expression for lymphoid and myeloid leukemias, respectively. All patients' samples were obtained from the Clinical Hematology Department of the Academic Hospital of the Vrije Universiteit Brussel, Brussels and the Université Catholique de Louvain, Brussels, Belgium.

Control hematopoietic cells

Normal blood or bone marrow was obtained from blood donors and from allogeneic bone marrow donors at the time of collection. The institutional Ethics Committee approved this study. These samples were used to isolate normal CD19+ B cells and normal CD33+ progenitors to serve as reference material for the flow cytometry studies. Mononuclear cells from peripheral blood and bone marrow were separated by Ficoll-Hypaque density gradient centrifugation (Lucron Bioproducts, De Pinte, Belgium) The isolated cells were further washed and diluted in PBS Facsflow (Becton Dickinson) supplemented with 0.5% BSA.

Flow cytometry

Indirect immunofluorescence (IIF) staining of cells was performed as follows. Twenty-five µls of a cell suspension ($4x10^6$ cells/ml of PBS supplemented with 5% BSA) were incubated for 20 min at room temperature with 25 µls of a mAb preparation. Following three washings with PBS supplemented with 1% BSA, 500 µL of FACS flow solution was added (Becton Dickinson) and samples were analyzed. Data were acquired, analyzed and displayed by a Coulter Epics II – MCC cytometer using the Coulter System II – software v 3.0 (Coulter, Miami, FL, USA). The mean fluorescence intensity (MFI) from samples was noted and used for further analysis.

Magnetic cell sorting system (MACS)

The MACS-system and the microbeads were obtained from Miltenyi (Sanvertech, Boechout, Belgium) and applied as described by the manufacturer. In brief, a starting population of mononuclear cells was incubated for 15 min with the selected mouse anti-CD antibodies, as indicated later. Cells were then washed in buffer and incubated for 15 min on ice in 100µL of a 1:10 dilution of the anti-mouse IgG coated magnetic microbeads.

The cell suspension was then applied to a MIDIMACS column in a magnetic collar. The column was washed thrice to obtain untouched cells. Bound cells (touched fraction) were

recovered by removing the magnetic collar and passing 5 mL of buffer through the column using a plunger.

Negative (untouched) isolated cell suspensions were utilized in all experiments to study HLA antigen expression. This strategy was utilized to exclude the possible interference of antibody-coated magnetic beads retained on cells in additional assays. Moreover, HLA antigen expression on leukemic cells and autologous normal cells could be studied under identical experimental conditions.

Therefore, two individual magnetic bead separations were performed on each peripheral blood and bone marrow sample to obtain untouched normal and leukemic cell fractions. Different microbeads were used depending on the tumor type.

To isolate leukemic B cells from CLL-B or ALL-B samples the patients' normal cells were magnetically labeled by a cocktail of anti-CD2, -CD4, -CD11b,-CD16 and -CD36 mAb. Unlabeled CD19+ B cells were recovered by washing. Patients' normal cells were obtained after the malignant B cells were retained on the column by anti-CD19 microbeads. In the case of myeloid tumors patients' normal cells were isolated after tumor cells were bound to the column through anti-CD33 microbeads. Untouched CD33+ tumor cells were obtained by retaining normal cells on the column by a combination of anti-CD2 and anti-CD19 microbeads. The purity of isolated cells, as tested by flow cytometry, was always higher than 95 %.

All touched, positive fractions (patients' leukemic and normal cells) were cryopreserved in complete medium containing 10% DMSO for eventual DNA isolation purposes. An identical procedure was used to isolate normal B cells and normal T cells from peripheral blood and bone marrow from healthy donors. No attempt was made in this study to isolate the small fraction of NK cells (5%) out of the major T-cell fraction (95%). These cell preparations are called "normal cells" in the present study.

Purified, untouched cell preparations were preferentially used for flow cytometry studies. The remaining cell preparations were used for CDC assays and in part cryopreserved for staining experiments with HLA class I allele specific mAb.

Serological HLA typing

The HLA-A and HLA-B phenotype was established on the different isolated cell fractions using the conventional CDC assay. Each typing plate contained at least two sera identifying individual HLA-class I allospecificities. When weak or extra reactions were obtained, cell preparations were tested with additional commercially available, typing plates (Biotest Seralc, Kortenberg, Belgium). All microcytotoxicity assays were supervised by the same experienced technician ensuring reproducibility and reliability of the assays during the study.

Molecular HLA typing

In all cases in which typing by serology identified homozygosity for HLA-A or -B antigens, molecular typing was performed utilizing the reverse hybridization technique (Innogenetics, Ghent, Belgium). DNA was isolated using a routine salting-out method (DNA E-Z Prepkit, Orchid Diagnostics Europe, St Katelijne Waver, Belgium).

In the cases in which total HLA class I antigen loss was identified on leukemic cells by CDC assay, DNA was extracted from cryopreserved normal and leukemic cells (touched fractions). A 2 KB PCR amplicon spanning exons 1 to 5 from HLA-A and -B antigens was generated using a Gene Amp PCR 9700 (Applied Biosystems, Foster City, CA, USA). The PCR product was cleaned-up using Centricon YM-100 filters (Millipore, Brussels, Belgium) and visualized in agarose gel electrophoresis using ethidiumbromide. Sequencing reactions were performed separately for exons 2, 3 and 4 both in forward and reverse directions for each sample using Big Dye Terminator Chemistry (Applied Biosystems). Sample electrophoresis was done on an ABI Prism 310 sequencer. Sequencing reactions were analyzed using ABI Match Tools PPC v 1.0. Both the PCR amplification and sequencing reagents were purchased from Applied Biosystems.

Statistics

The non-parametric (two-sided) Mann-Whitney U test was used to calculate probabilities between groups. $P \leq 0.05$ was considered significant.

Results

Specificity of human anti-HLA class I mAb

Preliminary experiments analyzed the specificity of the panel of human mAb to be utilized in this study by testing with a panel of HLA class I typed peripheral blood lymphocytes and cultured lymphoid cells. The results are shown separately for HLA locus A (Table 1) and locus B (Table 2) antigen-specific mAb. The reactivity of mAb with cells expressing the corresponding HLA class I allospecificity was 1 to 3 log stronger than that with negative controls in flow cytometry. Representative results are shown in Fig 1a, b. Overlapping profiles were not observed with any of the mAb. Some mAb recognize determinants expressed by more than one HLA class I allospecificity. For example, mAb SN66E3 recognizes a determinant shared by HLA-A2 and A28 allospecificities. In these cases, target cells were selected for expressing only one of these allospecificities. Other mAb, such as clone VDK1D12, are restricted in the recognition of only one HLA class I allospecificity. The overall results showed that all mAb, except mAb BR011F6, reacted as predicted by the HLA type of all the cells under study confirming their specificity. mAb BR011F6 did not recognize HLA-A1104. All mAb did not stain the HLA class I negative K562 cells.

Table 1: Reactivity of HLA-A specific HumAb with a panel of HLA typed peripheral blood lymphocytes and cell lines [a]

mAb	Isotype	Specificity	PBL	Cell lines	Flow cytometry
VDK1D12	IgM, κ	A1	10[b]	2[b]	+++
SN66E3	IgM, κ	A2	22	3	++/+++
		A28	3	0	+++
OK2F3	IgM, κ	A3	11	2	+++
GV5D1	IgG1, λ	A1	10	2	+++
		A9	8	0	+++
NIE44B8	IgM, κ	A10	4	1	+/++
BRO11F6	IgG1, λ	A11	4*	3	-/+++
HDG6B6	IgM, λ	A29,31,32,33 (19)	12	2	+
SN230G6	IgG1, λ	A2	22	3	+++
OK5A3	IgM, λ	A3	8	2	+++
		A11	3	3	+
		A1	10	2	+
OK4F9	IgM, κ	A1	8	2	+++
		A3	7	2	+++
		A11	3	3	+++
HDG2G7	IgG1, κ	A29,31,32,33 (19)	16	2	+++
OK1C9	IgM, λ	A3	11	2	+++

A11	3	2	+
A33 (19)	2	0	+
A31 (19)	2	1	+

[a] HLA-A allele specific mAb were tested with HLA class I typed PBL and cell lines in IIF. Two isotype-matched controls were included in every experiment. With the exception of mAb BRO11F6, all mAb stained all cell samples expressing the corresponding HLA class I allospecificity. (*) mAb BRO11F6 stained only 2 out of the 4 HLA-A1104 positive PBL samples. Reactivity is expressed as + (1 log), ++ (2 logs), +++ (3 logs) stronger than the negative controls used.

[b] number of samples tested

Table 2: Reactivity of HLA-B specific HumAb with a panel of HLA typed lymphocytes and cell lines [a]

mAb	Isotype	Specificity	PBL	Cell lines	Flow cytometry
13E12	IgM, κ	B12	7 [b]	0 [b]	+++
GK31F12	IgM, κ	B13	4	0	+++
HA2C10B12	IgM, κ	B60(40)	7	2	+++
VTM3A1	IgG1, κ	B7	7	4	+++
VTM4D9	IgG1, κ	B7	7	4	++
DMS4G2	IgG1, λ	B62(15)	2	2	++/+++
		B35	7	2	+++
KAL3D5	IgG1, λ	B51(5)	5	2	+++
HDG8D9	IgG1, λ	B51(5)	5	2	+++
GR5B3	IgM, λ	B62 (15)	2	2	++
BVK5B10	IgM, κ	B8	7	1	+++
AE9D9	IgM, λ	B8	7	1	+
		B14	3	0	0
OK6H10	IgM, κ	B15	1	2	+++
		B35	7	2	+++
SN230G6	IgG1, λ	B17	3	0	+++
KG30A7	IgM, λ	B12	7	1	++
		B14	4	0	+++
GVK2F8	IgM, λ	B18	4	1	+++
		B39 (16)	3	0	+++
HDG2G7	IgG1, κ	B57 (17)	2	0	+++
OK6H10	IgM, κ	B35	7	2	+++
		B15	1	2	+++
		B15	1	2	+++
		B35	7	2	+++
FVS4G4	IgM, κ	B35	7	2	+++
		B17	6	0	+
		B62(15)	2	2	+++
		B51(5)	5	2	+++
		B14	5	0	+++
		B18	4	1	++/+++
		B38(16)	3	1	+++

[a] HLA-B allele-specific mAb were tested with HLA class I typed PBL and cell lines in IIF.

Two isotype-matched controls were included in every experiment. All mAb stained all cell samples expressing the corresponding HLA class I allospecificity. Reactivity is expressed as + (1 log), ++ (2 logs), +++ (3 logs) stronger than the negative controls used.

[b] number of samples tested

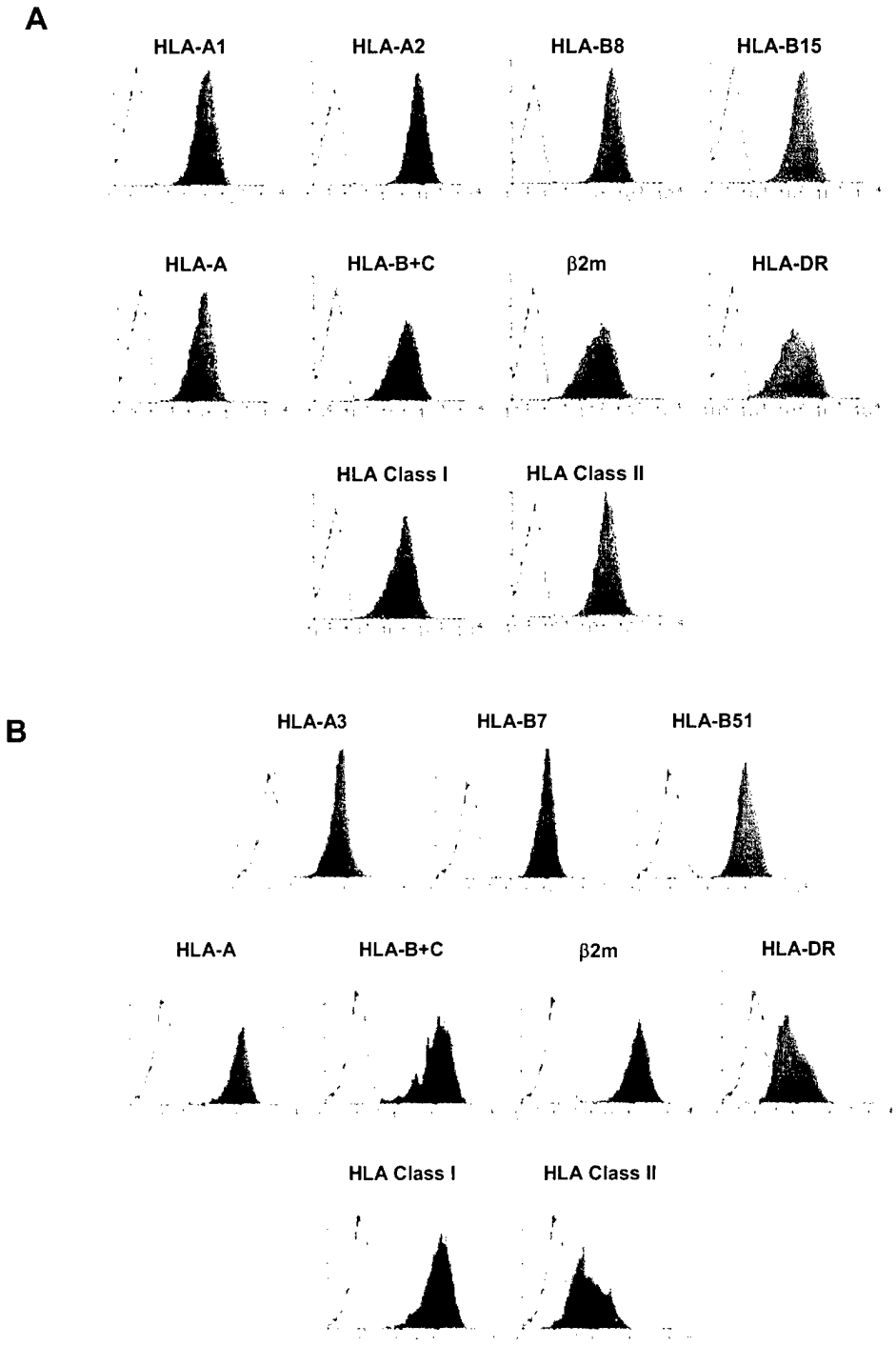

Figure 1. HLA antigen expression by cell lines

Flow cytometric analysis of NALM-6 (Figure 1a) and MONO-MAC-6 (Figure 1b) cell lines stained with HLA class I and class II antigen-specific mAb (black) Irrelevant, isotype-matched mAb were used as controls (white).

Changes in HLA class I antigen level on leukemic cells

In an initial study HLA class I antigen level on leukemic cells from 17 CLL-B, 9 ALL-B and 20 AML patients was analyzed by flow cytometry utilizing the anti-HLA-A, B, C mAb W6/32, the anti-HLA-A mAb LGIII-147.4.2 and the anti-HLA-B mAb B1.23.2. The expression level of HLA class I antigens on untouched leukemic B cells was compared to that on HLA matched B cells from healthy donors. Total HLA class I antigen level, as measured with the mAb W6/32, was significantly lower (p=0.0067) on CLL-B cells than on normal B cells (Table 3). A difference was also found, when cells were stained with HLA locus-specific mAb. However this difference did not reach the level of statistical significance. Total HLA class II antigen level was also significantly lower (p=0.0134) on malignant B cells than on normal B cells.

Table 3: HLA antigen expression on CLL-B and ALL-B cells [a]

	Normal B-cells MFI (SD) (n=10)	CLL-B MFI (SD) (n=17)	p-value	ALL-B MFI (SD) (n=9)	p-value
HLA-A, B, C	87 (33)	55 (39)	0.0067	32 (24)	0.0012
HLA-A	28 (8)	22 (16)	NS	16 (8)	0.0002
HLA-B	33 (14)	32 (14)	NS	16 (10)	0.005
β2m	69 (32)	52 (37)	NS	24 (15)	NS
HLA-DR, DQ, DP	30 (7)	17 (13)	0.0134	22 (23)	NS
HLA-DR	29 (2)	19 (15)	0.0452	26 (20)	NS

[a] CLL-B and ALL-B cells at diagnosis and peripheral blood B cells isolated from healthy donors were stained with anti-HLA mAb and analyzed by flow cytometry. All cells tested were untouched fractions derived from immunomagnetic isolation procedures. Results are expressed as mean fluorescence intensity values + SD. Results obtained with leukemic and normal B cells were compared utilizing the non-parametric Mann-Whitney U test.

The decrease in total HLA class I antigen level on ALL-B cells was even more pronounced than on CLL-B cells. In all individual cases (data not shown) a severe decrease of HLA-A and -B antigens was observed. Furthermore, in all CLL-B and ALL-B cases malignant cells expressed β2m. On the other hand no significant changes were noted for HLA class II antigen expression.

In contrast, HLA class I antigen level on CD33+ AML cells was significantly increased when compared to HLA matched $CD33^+$ bone marrow derived progenitors from healthy donors (Table 4). Also in this case, the difference between leukemic cells and normal counterparts

did not reach the level of statistical significance when cells were stained with HLA locus specific mAb. In all AML cases malignant cells expressed β2m. HLA class II antigen expression, and especially HLA-DR antigen expression was increased significantly.

Table 4: HLA antigen expression on bone marrow AML and $CD33^+$ normal donor progenitors

	AML MFI (SD) (n=20)	$CD33^+$ normal progenitors MFI (SD) (n=10)	p-value
HLA-A, B, C	53 (32)	15 (11)	0.0032
HLA-A	32 (22)	19 (10)	NS
HLA-B	41 (26)	26 (15)	NS
β2	62 (52)	21 (19)	0.0119
HLA-DR,DQ,DP	28 (31)	7 (2)	0.0329
HLA-DR	33 (31)	6 (2)	0.0013

AML cells at diagnosis and HLA matched bone marrow derived CD33+ progenitors from healthy donors were stained with anti-HLA mAb and analyzed by flow cytometry. All cells tested were untouched CD33+ cells obtained from immunomagnetic isolation procedures. Results are expressed as MFI values ± SD. Results obtained with AML cells and progenitors from healthy donors were compared utilizing the now-parametric Mann-Whitney U test.

Selective HLA class I antigen loss by leukemic cells

Immunomagnetically separated normal and leukemic cells from 37 leukemic samples were analyzed for HLA class I antigen expression by CDC (Table 5). Representative results obtained with two samples are shown in Fig. 2. HLA class I antigen defects were found in 5 samples. Specifically HLA-A locus antigen loss was found in 1 sample, loss of one HLA-A allospecificity in two samples and loss of one HLA-B allospecificity in two samples.

To determine whether the detection of only one HLA-B allospecificity in case 21 reflected homozygosity at the HLA-B locus or loss of one HLA-B allospecificity molecular typing of leukemic cells was performed. This sample was found to be homozygous at the HLA-B locus. In 5 samples too few normal cells could be isolated to perform serological HLA typing.

Table 5: Selective HLA class I antigen loss identified by testing leukemic cells with HLA class I antigen typing sera in CDC

Case No	Leukemic Subtype	Sample type	Normal Cells				Tumor Cells			
			A	A	B	B	A	A	B	B
1	CLL	BL	10	31	38	40	■	■	38	40
2	AML	BL	24	31	60	-	24	31	60	-
3	CLL	BL	2	3	7	51	2	3	7	51
4	CLL	BL	1	2	5	8	1	2	5	8
5	CLL	BL	3	10	35	44	3	10	35	44
6	AML	BL	24	32	39	57	24	32	39	57
7	CLL	BL	2	10	8	14	2	10	■	14
8	ALL	BL	2	3	60	-	2	3	60	-
9	CLL	BL	1	3	35	40	1	3	35	40
11	AML	BL	1	2	7	60	1	2	7	60
12	CLL	BL	3	23	7	44	3	23	7	44
13	CLL	BL	1	24	8	18	1	24	8	18
14	CLL	BL	24	32	35	51	24	32	35	51
15	AML	BL	ND	ND	ND	ND	3	11	35	38
16	CLL	BL	2	24	13	18	2	24	13	18
17	ALL	BL	1	-	5	7	1	-	5	7
18	ALL	BL	2	33	7	14	2	33	7	14
21	ALL	BL	ND	ND	ND	ND	10	28	52	-
23	AML	BL	2	32	8	17	2	32	8	17
26	CLL	BL	2	-	7	44	2	-	7	44
27	CLL	BL	2	-	8	44	2	-	8	44
28	CLL	BL	2	32	18	35	2	32	18	35
29	AML	BL	2	28	13	18	2	■	13	18
30	AML	BL	2	-	17	42	2	-	17	42
31	CLL	BL	11	33	35	57	11	33	35	57
32	AML	BL	2	3	39	51	2	3	39	51
33	AML	BL	2	23	35	44	2	23	35	44
34	AML	BM	1	2	13	60	1	2	13	60
38	CLL	BL	1	2	8	12	■	2	8	12
40	AML	BL	3	29	44	55	3	29	44	55
41	CLL	BL	3	29	51	60	3	29	51	60
42	CLL	BL	2	19	39	-	2	19	39	-
43	AML	BL	ND	ND	ND	ND	11	24	35	62
44	AML	BL	2	29	35	51	2	29	■	51
46	AML	BL	ND	ND	ND	ND	3	25	22	35
47	CLL	BL	1	3	7	8	1	3	7	8
48	AML	BL	ND	ND	ND	ND	1	2	8	60

Untouched leukemic cells and autologous lymphocytes were isolated using immunomagnetic procedure. HLA class I allospecificity expression was assessed by testing cells with HLA class I typing sera in CDC and tested for HLA class I expression by CDC.(-: homozygous sample; ND: not done, not enough normal cells available; ■: no reaction with the respective anti-HLA class I antisera).

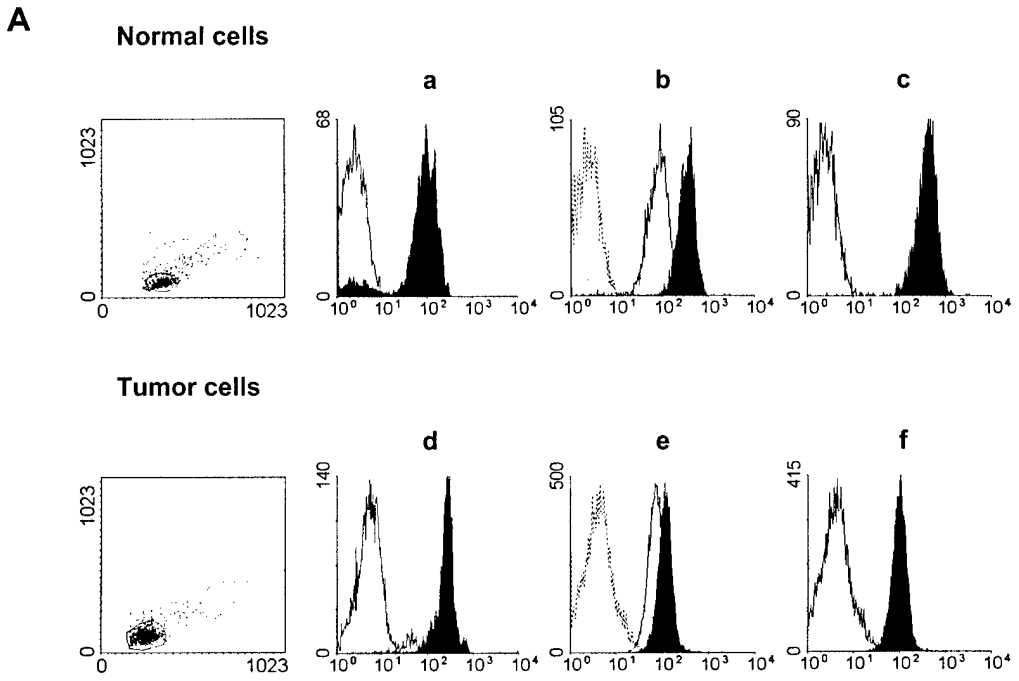

Figure 2. HLA class I antigen expression by immunomaganetic isolated lymphoid leukemic and autologous normal lymphocytes.
Untouched leukemic cells from a CLL-B patient (bottom row) were stained with anti-CD3 (white) and anti-CD19 (black) mAb, (panel d), anti-HLA-A mAb LGIII-147.4.2 (white), anti-HLA-B mAb B1.23.2 (black) and control mAb (dotted) (panel e) and with anti-HLA-A, B, C mAb W6/32 (black) and with control mAb (white) (panel f). Untouched autologous normal cells (top row) were stained with anti-CD3 (black), and anti-CD19 (white) mAb (panel a), anti-HLA-A mAb LGIII-147.4.2 (white), anti-HLA-B mAb B1.23.2 (black) and control mAb (dotted) (panel b) and with anti-HLA-A, B, C mAb W6/32 (black) and control mAb (white) (panel c). Forward/side scatter dot plots of the isolated cell populations are shown on the left.

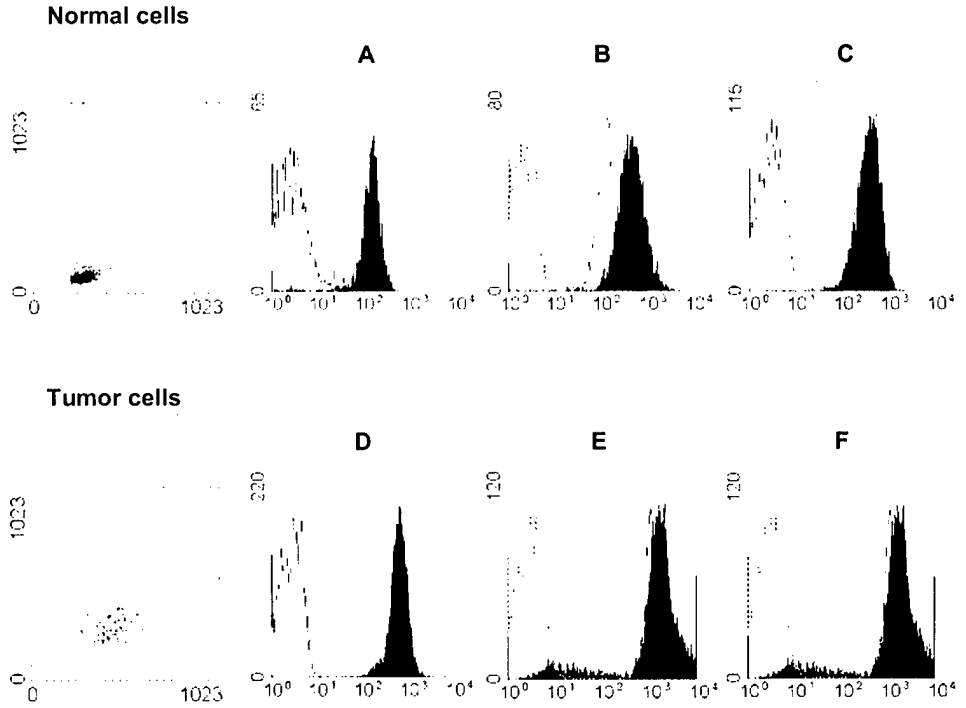

Figure 3. HLA class I antigen expression by immunomagnetic isolated myeloid leukemic and autologous normal myeloid cells.
Untouched leukemic cells from an AML patient (bottom row) were stained with anti-CD3 (white) and anti-CD33 (black) mAb, (panel d), anti-HLA-A mAb LGIII-147.4.2 (white), anti-HLA-B mAb B1.23.2 (black) and control mAb (dotted) (panel e) and with anti-HLA-A, B, C mAb W6/32 (black) and with control mAb (white) (panel f). Untouched autologous normal cells (top row) were stained with anti-CD3 (black), and anti-CD33 (white) mAb (panel a), anti-HLA-A mAb LGIII-147.4.2 (white), anti-HLA-B mAb B1.23.2 (black) and control mAb (dotted) (panel b) and with anti-HLA-A, B, C mAb W6/32 (black) and control mAb (white) (panel c). Forward/side scatter dot plots of the isolated cell populations are shown on the left.

Confirmation of HLA-A and -B allelic losses on leukemic cells by flow cytometry

The availability of HLA class I allele-specific mAb allowed us to study the HLA antigen expression on leukemic cells by flow cytometry. Cryopreserved normal and leukemic cells were available from 4 samples in which single HLA class I allele loss had been detected by CDC. mAb recognizing all the HLA-A and -B allospecificities expressed in the four samples were available to us and were therefore utilized to stain leukemic cells isolated from the 4 samples. As shown in Fig. 4, HLA-A10 and A31 alleles were not detected in sample 1 and HLA-B8, HLA-A28 and HLA-A1 alleles were not detected in samples 7, 29 and 38, respectively. The contour plots are identical to those from the negative isotype controls.

Furthermore HLA-B8 allele was barely detectable by flow cytometry on leukemic cells from sample 38, although it was easily detectable by CDC. This finding illustrates the high sensitivity of CDC for HLA typing and that the latter methodology is useful to detect total and selective HLA class I losses, but is not useful to measure HLA class I antigen downregulation. HLA-B8 allele expression is approximately 10-times lower (1 log) on leukemic cells than on autologous normal cells. It is noteworthy that in this sample HLA-A2 allospecificity was not detectable on a small fraction of leukemic cells. This finding may reflect the emergence of a variant leukemic clone with HLA-A2 antigen loss. Total HLA class I antigen losses were not detected on patients' normal cells emphasizing the association of HLA class I antigen defects with malignant transformation of cells.

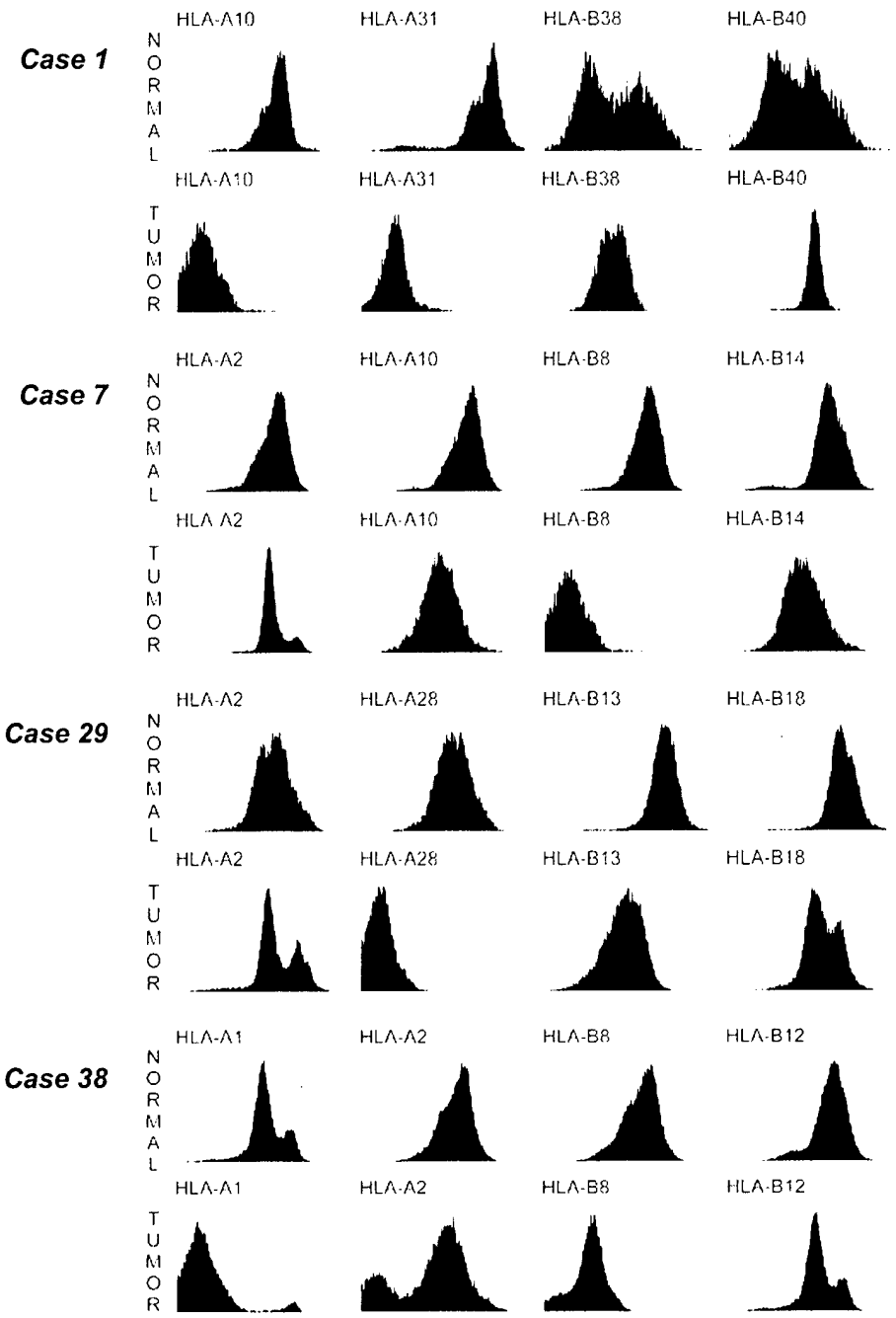

Figure 4. Detection of selective HLA class I allelic losses on leukemic cells by flow cytometry analysis. Leukemic and autologous normal cells were stained with HLA class I allele-specific mAb (black) and analyzed by flow cytometry. Cells stained with irrelevant isotype matched mAb were used as controls (white).

Characterization of mutations underlying selective HLA-A and B allele losses in leukemic cells

To define the molecular mechanism(s) underlying HLA class I allele losses by leukemic cells, the HLA-A genes in samples 1, 29 and 38 and the HLA-B gene in sample 7 were cloned from leukemic and autologous normal cells. Exons 2, 3 and 4 of each gene were sequenced in forward and reverse direction. No mutation was detected in the three exons in the 4 samples analyzed.

HLA class I allele expression levels on leukemic cells

Additional experiments took advantage of the panel of HLA class I allele-specific human mAb available to us to compare the expression level of HLA class I alleles on leukemic and autologous normal cells. In these experiments the level of HLA class I alleles on leukemic cells was not compared with that on allogeneic normal lymphoid cells to avoid the interference of individual genetically determined variability in HLA class I antigen expression with the interpretation of the results. The results obtained with leukemic and normal cells are expressed as MFI. They are presented separately for the gene products of HLA-A and-B loci in Table 6. A ratio R value was calculated utilizing the formula R=MFI leukemic cells/MFI T cells. A HLA class I allele was classified as downregulated when the R-value was lower than 1. T cells and not total lymphocytes were used for comparison since in preliminary studies total HLA class I antigen expression as well as individual HLA class I allele expression was found to be lower on T cells than on autologous B cells isolated from healthy donors. The mean MFI value obtained with B cells was approximately double that obtained with autologous T cells.

Of the 56 HLA-A and 50 HLA-B alleles analyzed in 32 samples, 20 (35%) and 19 (38%), respectively, were found to be downregulated on leukemic cells. Combining the results obtained with HLA-A and B alleles, one or more HLA class I alleles were found to be downregulated in 21 (65%) out of the 32 samples analyzed. This is likely to be an

underestimated value, since the expression of some HLA-A and -B alleles present in the patients' HLA class I phenotype could not be investigated because of the lack of availability of mAb with the appropriate specificity.

Table 6: HLA-A and –B allospecificity expression on leukemic cells.

		HLA LOCUS A				HLA LOCUS B			
Case no	Leukemic subtype	HLA allele	Normal T cells	Malignant cells	R	HLA allele	Normal T cells	Malignant cells	R
3	CLL-B	A2	41.0	69.3	1.69	B7	63.8	57.7	**0.90**
		A3	92.5	184.9	1.99	B51	120.4	156.2	1.29
4	CLL-B	A1	20.7	19.8	**0.95**	B5	ND	ND	-
		A2	39.9	33.9	**0.84**	B8	59.3	57.7	**0.97**
13	CLL-B	A1	23.9	42.4	1.77	B8	83.1	43.7	**0.52**
		A24	29.2	38.6	1.32	B18	24.5	93.4	3.81
27	CLL-B	A2	30	18.2	**0.60**	B8	56.7	37.2	**0.65**
		H			-	B44	ND	ND	-
28	CLL-B	A2	29.2	31.9	1.09	B18	6.14	28	4.56
		A32	ND	ND	-	B35	57.2	36	**0.62**
29	AML	A2	21.1	14.1	**0.66**	B13	10.5	10.7	1.01
		A28	18.2	2.83	**0.15**	B18	2.14	1.97	**0.92**
30	AML	A2	16.8	10.9	**0.64**	B17	27.9	16.7	**0.59**
		H			-	B42	ND	ND	-
31	CLL-B	A1	25.7	29.0	1.12	B35	17.7	17.3	**0.97**
		A11	42.7	46.7	1.09	B57	38.6	39.1	1.01
32	AML	A2	29.1	29.6	1.01	B39	4.9	7.1	1.44
		A3	12.2	17.6	1.44	B51	34.7	47.3	1.36
33	AML	A2	7.6	13.9	1.82	B35	14.9	25.8	1.73
		H			-	H			-
34	AML	A1	57.3	78.1	1.36	B13	44.6	58.7	1.31
		A2	87.7	126.6	1.44	B60	12.1	20.1	1.66
43	AML	A3	6.46	7.33	1.13	B35	14.8	4.63	**0.31**
		A11	5.53	7.45	1.34	B62	15.4	6.85	**0.44**
45	AML	A1	19.2	5.98	**0.31**	B7	11.8	8.33	**0.70**
		A2	34.6	6.05	**0.17**	B60	9.46	8.18	**0.86**
50	ALL-B	A3	4.91	17.7	3.60	B7	12.1	23.0	1.9
		A26	5.07	17.9	3.53	B38	ND	ND	-
54	CLL-B	A1	20.1	6.79	**0.33**	B8	8.31	10.2	1.22
		A28	12.5	6.57	**0.52**	B49	ND	ND	-
55	CLL-B	A3	8.53	11.7	1.37	B7	19.3	10.8	**0.55**
		A33	ND	ND	-	B35	32.0	29.3	**0.91**
56	CLL-B	A2	32.5	4.75	**0.14**	B7	16.9	5.24	**0.30**
		A33	ND	ND	-	B60	12.9	10.5	**0.81**
57	AML	A3	7.05	7.89	1.11	B35	5.84	7.94	1.35
		A11	8.25	9.18	1.11	B39	ND	ND	-
61	CLL-B	A2	67.4	41.4	**0.61**	B13	70.9	64.0	**0.90**
		A3	15.9	7.8	**0.49**	B61	ND	ND	-
62	CLL-B	A32	6.1	10.5	1.72	B62	52.6	45.5	**0.86**
		H			-	H			-
63	CLL-B	A2	37.4	22.1	**0.59**	B44	7.4	7.1	**0.96**
		A31	3.7	3.1	**0.84**	B18	5.2	6.1	1.17
64	CLL-B	A2	50.3	37.3	**0.74**	B15	35.4	39.9	1.13
		A24	49.2	34.1	**0.69**	H			-
65	CLL-B	A32	37.2	61.0	1.64	B13	46.8	103.8	2.21
		A9	11	26.6	2.42	B39	2.8	13.3	4.75
67	CLL-B	A2	26.5	35.5	1.34	B8	50.5	212.5	4.21
		A24	20.6	137.5	6.67	B44	7.1	117.6	16.56
68	CLL-B	A28	45.1	40.8	**0.90**	B53	ND	ND	-
		A32	4.7	27.3	5.81	B7	23.5	43.1	1.83
69	CLL-B	A3	22.3	27.8	1.25	B35	17.1	28.6	1.67
		A10	4.2	5.9	1.40	B44	5.4	5.9	1.09
70	CLL-B	A26	90.1	64.4	**0.71**	B18	ND	ND	-
		H				B55	ND	ND	-
71	CLL-B	A1	31.9	43.9	1.38	B50	33.9	61.3	1.81
		A24	51.3	81.9	1.60	B62	59.9	87.9	1.47
72	CLL-B	A2	30.5	33.3	1.09	B35	34.4	49.6	1.44
		A3	22.8	25.0	1.10	B60	12.3	20.6	1.67
73	CLL-B	A11	43.1	25.2	**0.58**	B14	60.5	75.3	1.24
		A19	36.6	39.4	1.08	B51	30.2	36.5	1.21

74	CLL-B	A1	25.8	29.4	1.14	B8	55.9	57.0	1.02
		A2	36.4	34.2	**0.94**	B51	41.4	42.0	1.01
75	CLL-B	A2	20.3	29.6	1.46	B39	6.9	19.6	2.84
		A31	3.8	8.8	2.32	H			-

HLA class I allelic expression levels were determined by flow cytometry on leukemic cells and on autologous normal T cells. All cells tested were untouched cell fractions obtained by immunomagnetic procedures. The results are expressed as MFI after background correction. The ratio R was derived from MFI malignant cells / MFI normal cells. An R value lower than 1 was considered as downregulation of that particular allele (H: homozygous; ND: not done, no specific antibody available).

Restriction of HLA class I antigen downregulation to HLA-Bw6 allospecificities

HLA class I allele expression on leukemic cells was further analyzed taking into account whether they belong to the HLA-Bw4 or Bw6 group. Out of the 36 HLA-B alleles belonging to the HLA-Bw6 group, 16 (44%) were downregulated. In contrast only 3 (14%) of the HLA-A and HLA-B alleles belonging to the HLA-Bw4 group were downregulated. The difference in the frequency of downregulation between HLA class I alleles belonging to the HLA-Bw6 group and the remaining HLA class I alleles is statistically significant ($p<0.03$). Downregulation of HLA-Bw6 antigens, at variance from that of HLA-Bw4 antigens, does not affect the interaction of target cells with NK cells.[26] Therefore our results suggest that emerging tumor cells acquire a HLA class I antigen phenotype which allows them to escape not only from CTL, but also from NK cells.

Discussion

The present study has analyzed for the first time HLA class I antigen expression on leukemic cells utilizing three types of probes. They include mouse mAb which recognize framework or locus specific determinants of HLA class I antigens and a large panel of conventional HLA typing sera and of humAb which recognize HLA class I alleles. Both CDC and flow cytometry have been used as assays. In agreement with the scanty information available in the literature.[13,14] HLA class I antigen loss has been detected in a very low percentage of leukemic samples. This finding is a variance with what has been found in most every type of solid tumor analyzed.[3,4] We believe that this difference is likely to reflect the shorter time between malignant transformation and diagnosis in leukemia than in solid tumors. A short time interval between the onset of leukemia and diagnosis may not be sufficient for cells to

acquire mutations in the gene(s) involved in HLA class I antigen expression and for selective pressure to facilitate the expansion of malignant cells with HLA class I antigen abnormalities. In our study we could not detect any abnormality in the sequence of the polymorphic exons of the lost HLA-A and -B antigens. The other exons and introns of these antigens could not be analyzed because of the lack of sufficient DNA which could be isolated from each patient. Therefore we cannot exclude abnormalities in these regions of the lost HLA class I genes. However the available information in the literature suggests that defects in regulatory mechanisms controlling HLA class I antigen expression and not structural abnormalities of HLA class I genes may be the underlying mechanism of HLA class I antigen loss in leukemic cells. Brouwer et al[13] didn't detect abnormalities in genes encoding HLA class I antigens lost by leukemic cells. Moreover, in their experiments IFN-γ could restore HLA class I antigen expression by leukemic cells as well as T-cell recognition and lysis of the target cells. Furthermore Real et al[27] showed that expression of a lost HLA class I allospecificity could be restored by treating leukemic cells with cytokines. We could not test whether the lost HLA class I allospecificities could be restored in leukemic samples by cytokines, since additional blood samples could not be obtained from the patients with a complete HLA class I allelic loss. All of them died shortly after diagnosis. Whether this association is a fortuitous one or reflects an aggressive phenotype of leukemic cells with HLA class I antigen loss and/or an escape of leukemic cells from CTL recognition and destruction remains to be determined, since the number of patients with this HLA class I phenotype we have identified in our studies is too small to draw conclusions.

The availability of a large panel of humAb recognizing HLA class I allospecificities has allowed us to analyze for the first time the level of HLA class I alleles on leukemic cells. Comparison of the expression level of 116 HLA class I alleles on 32 leukemic samples and autologous normal cells has detected downregulation of HLA-A and HLA-B alleles in 35 and 38%, respectively, of the samples analyzed. As a result downregulation of HLA-A and/or HLA-B allospecificities is present in the majority of the leukemic patients investigated. How these findings compare with the HLA phenotypes in solid tumors cannot be assessed at

present, since the methodology available at present is not suitable to measure HLA class I antigen level in solid tumors.

In our study downregulation does not affect all HLA class I alleles with similar frequency, but appears to be preferential for HLA class I alleles expressing the HLA-Bw6 determinant. This finding is intriguing, because of the differential interaction of HLA-Bw4 and Bw6 alleles with KIR receptors expressed on NK cells. In contrast to HLA-C allospecificities, not all HLA-B alleles inhibit NK cell cytotoxicity. Only the HLA-B alleles that carry the HLA-Bw4 epitope protect target cells from NK cell attach. Therefore it is tempting to speculate that the selective HLA-Bw6 downregulation provides leukemic cells with an escape mechanism from CTL attack. If this interpretation is correct, immunotherapeutic strategies should use HLA-Bw4 alloantigens as restricting elements since escape of leukemic cells from CTL, because of a HLA-Bw4 allele loss, would render them susceptible to NK cell mediated lysis.

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HLA antigen expression in breast cancer: a multicenter study utilizing formalin-fixed paraffinized tissues

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Abstract

Background

Immunohistochemical (IHC) staining of frozen tissues has shown that abnormalities in HLA class I antigen expression are frequent in malignant cells. Despite the possible clinical significance and potential for T-cell based immunotherapy, evaluation of malignant lesions for HLA class I antigen expression is not performed routinely, even for patients who are candidates for such therapy. This reflects, at least in part, reluctance by pathologists to utilize frozen tissue sections in IHC assays. Little information is available about the usefulness of formalin-fixed paraffin-embedded tissues (FFPT) as substrates in IHC assays to evaluate tissue expression of HLA antigens. We therefore undertook a multi center study to develop and standardize an IHC protocol using FFPTs and anti-HLA monoclonal antibodies (mAbs).

Methods

HLA class I and class II expression was examined in FFPT from three breast cancer patients. To determine if loss of expression of MHC Class I molecules at the protein level with IHC reflect alterations at the gene level, DNA from microdissected normal and tumor tissue was evaluated with microsatellites at the MHC class I 6p21 locus (HLA-A, B, C determinants) and at the 15q21 beta 2 microglobulin locus for loss of heterozygosity (LOH).

Results

HLA class I antigen down-regulation in conjunction with cellular heterogeneity of expression in three breast carcinoma cases was concordantly reported by the four participating laboratories with anti-HLA class I antibody HC-10 and with the anti-beta 2 microglobulin L368. Furthermore, the four laboratories detected no staining of normal or malignant breast tissue for the lesions stained with the anti-HLA class II LGII. In contrast, infiltrating lymphocytes were strongly stained by LGII. Downregulation of class 1 was reflected by LOH in cases 1 and 3 for the 15q21 locus and in case 1 at the 6p21 locus.

Conclusion

The results indicate that FFPTs represent a useful substrate to monitor HLA antigen expression in malignant lesions, especially when appropriate markers are used to differentiate malignant cells from lymphocytes and dendritic cells.

Introduction

HLA class I antigen expression in surgically removed malignant lesions has been extensively investigated in recent years (1-5). Frozen tissue sections have been used as a substrate in immunohistochemical reactions with mAbs because the determinants recognized by the large majority of them are not expressed in formalin fixed, paraffin embedded tissues. These studies have convincingly shown that abnormalities in HLA class I antigen expression are frequently associated with malignant transformation of cells (6-8). Furthermore studies of a limited number of patients have provided suggestive evidence that abnormalities in HLA class I antigens in malignant lesions may have a negative impact on the clinical course of the disease and on the outcome of T cell-based immunotherapy (2). In spite of this evidence analysis of HLA class I antigen expression in malignant lesions represents a criterion neither to evaluate patients with malignancies nor to select those to be treated with T cell-based immunotherapy. This phenomenon is likely to reflect, at least in part, pathologists' reluctance to use frozen tissue sections as a substrate in immunohistochemical reactions. To overcome this limitation in the routine analysis of HLA class I antigen expression in malignant lesions, the HLA and Cancer component of the 13th International Histocompatibility Workshop (May 2002, Victoria, British Columbia, Canada) made one of its goals the analysis of HLA class I antigen expression in formalin fixed malignant lesions and the assessment of its clinical significance.

Distinct mechanisms have been found to underlie the multiple tumor HLA class I immuno-surveillance escape phenotypes which have been identified by immunohistochemical staining of frozen tissue sections with mAbs which recognize monomorphic, locus-specific and allele-specific determinants of HLA class I antigens. HLA haplotype loss in tumor cells has been shown to be generated frequently by total or partial deletion of chromosome 6, which carries the major histocompatibility complex in humans (9-11). The detection of these losses has been facilitated by the recent identification of highly polymorphic microsatellite markers (short tandem repeats). Utilizing short tandem repeats mapped on chromosome 6 (6p21, HLA locus) and on chromosome 15 (5 q21 locus, β2 microglobulin), loss of heterozygosity differences have been described in the frequency of HLA class I genes and of β2m genes in various types of malignancies. The values range from about 15% in colorectal carcinoma lesions to about 50% in head and neck squamous cell carcinoma (9, 10) and in cervical carcinoma lesions (11). Whether these differences reflect the use of different markers, lack of standardization in the methodology used, and/or different characteristics of the malignancies and/or patients investigated remains to be determined.

The aim of the present study was to optimize and assess the reproducibility of the assays to measure the expression of HLA class I antigens and to detect LOH at the HLA locus (6p21) and at the β2m locus (15q21) in formalin fixed, paraffin embedded normal breast epithelium and corresponding malignant breast lesions.

Materials and Methods

Four laboratories participated in this evaluation (1-Garrido, 2-Tilanus, 3- Ferrone, 4-Worsham), using shared antibodies (prepared in lab 3) and sections cut from the same tissue blocks (prepared in lab 4).

Monoclonal antibodies and conventional antisera

The anti-HLA class I heavy chain mAb HC-10, the anti-β_2 microglobulin (β_2m) mAb L368 and the anti-HLA class II mAb LGII-612.14 (LGII) were developed and characterized as described (12-14). Peroxidase-conjugated anti-mouse IgG xenoantibodies were purchased from Vector Laboratories, Inc (Burlingame, CA).

IHC methodology, validated in Laboratory 4 with recommended dilutions for the three antibodies was provided to the other participating centers. In brief, dilutions were as follows: mAb HC-10: 1/100 dilution, mAb L368: 1/25, mAb LGII612.14: 1/25, with overnight incubation for all three. The standardized methodology utilizes antigen-retrieval with endogenous peroxidase inactivation using 3% hydrogen peroxide, with normal horse serum as the blocking agent (Vector Laboratories Inc.). Following incubation with monoclonal dilutions, the slides are washed and incubated with biotinylated horse anti-mouse immunoglobulin G (Vector Laboratories, Inc.), followed by incubation with avidin-biotin peroxidase complex (Vectastain Elite ABC Kit: Vector Laboratories, Inc.). Immunoreactivity is visualized with 3', 3-diaminobenzidine tetrahydrochloride (Vector laboratories, Inc.) and the sections are counterstained with Mayer's Hematoxylin.

IHC Interpretation

Scoring of stain intensity in the tumor areas were performed with reference to staining intensity of normal breast tissue (control) present either in the same section with the breast tumor, or processed concurrently with the tumor section. Stromal lymphocytes in lymphocytic infiltrates surrounding the tissue, which stain intensely, are also considered as a reference for normal staining intensity.

Scoring parameters included cell membrane localization. Staining interpretation is derived as follows: 0-25% of cells = negative; >25%-<50% = heterogeneous; >50%-<75% = heterogeneous; (>25%-<75% = heterogeneous); >75% = positive staining. Scoring was done by two independent reviewers in each of the four centers in a blinded fashion. Complete absence of staining as compared to the presence of staining in the normal breast epithelium and in surrounding lymphocytes is scored as "complete loss". Marked decrease in staining intensity is scored as "+", presence of some staining intensity receives a score of "++", a score of "+++" indicates intensity of stain seen in normal breast tissue or surrounding lymphocytes.

Molecular methods: loss of heterozygosity

Processing Tumor Specimens for DNA Analysis

DNA from normal and tumor tissue was extracted from 4-5 micron sections from archival paraffin embedded tissue blocks. DNA was extracted using the QIAamp Kit (Qiagen Inc. Chatsworth, CA) according to the manufacturer's protocol.

Microsatellite analysis

A minimal microsatellite panel to detect LOH at the HLA locus at 6p and the β_2m locus at 15q included D6S1618, D6S291, D6S273, D6S265 and D6S311, and D15S126 and D15S209, respectively (Table 1). Fluorescent-labeled primers were obtained from Perkin-Elmer Applied Biosystems (Foster City, CA). 2µl-4µ of purified paraffin DNA from a total elution volume of 200ul from normal paraffin tissue and tumor in each case was used for each PCR reaction. PCR was performed in a 1X PCR buffer (10mM Tris-HCL pH 8.3, 50 mM KCl), 2.5mM $MgCl_2$, 200 uM each dNTPs, 1uM of each primer and 0.8 units of Ampli Taq Gold polymerase (Perkin-Elmer) in a 10ul reaction volume, using a Perkin Elmer GeneAmp 9600 followed by 35 cycles, 12 min 95 °C initial denaturation, an additional 50sec denaturation at 94 °C, 60 sec annealing at

57 °C and 60 sec extension at 70 °C. Amplification was completed with a final incubation step at 72 °C for 30 minutes.

The amplified PCR products were analyzed using an automated ABI PRISM sequencer (model 310, PE Applied Biosystems), using GeneScan Rox 400 size standard (PE Applied Biosystems). In brief, 12 ul deionized formamide were combined with 0.5 ul GeneScan Rox 400 size standard (PE Applied Biosystems) and 2ul of PCR product in a Genetic Analyzer sample tube. The tubes were closed with Genetic Analyzer septa and, after short mixing (vortex), the samples were denatured in a heat block for 4 minute at 90 °C, chilled on the ice, and spun briefly in a microcentrifuge in order to collect the contents. The samples were loaded on the ABI 310 genetic Analyzer and the run in accordance with the manufacturer's protocol (15).

Interpretation Criteria

A sample was defined as heterozygous if there were two distinct alleles for a given marker, i.e., maternal and paternal alleles were distinguishable (informative for LOH). The presence of only one size allele for a given marker, i.e., maternal and paternal alleles are indistinguishable (uninformative for LOH) was interpreted as homozygous. Loss of one of the two constitutive alleles in the tumor was scored as loss of heterozygosity (LOH). LOH was assigned when the fluorescent signal of one of two alleles was reduced by more than 30% as compared with the heterozygous control normal DNA sample (Figure 1).

RESULTS

Laboratory 2 employed a negative control and followed the provided protocol and dilutions exactly. Laboratory 1 made several variations to the provided protocol. Changes included the following: no avidin/biotin block, antigen retrieval was accomplished with the vector antigen retrieval solution in the microwave, and CD45 staining was also done to confirm the presence of lymphocytes and dendritic cells in tumor infiltrates.

HLA expression

In Case 1, for HC-10, among the 4 laboratories, expression was concordantly noted as heterogeneous in the tumor (Figure 2, Table 2). Heterogeneity of staining was attributed to varying degrees of tumor differentiation and also due to the presence of heavy lymphocytic infiltrate and CD45-positive dendritic cells within tumor areas, which resulted in an increased staining intensity in the tumor when compared with the normal/benign epithelium. For L-368, normal ducts were scored as negative for Case 1 by Laboratories 1 and 2 and heterogenous by Laboratories 3 and 4. For the tumor, when compared to staining intensities of stromal and lymphoctyic cells, all 4 laboratories noted downregulation for L-368. With LGII, agreement among the four centers was complete.

For case 2, for HC10, Laboratories 1, 2 and 3 scored normal epithelium as positive, with a heterogeneous interpretation by Laboratory 4. All four laboratories scored tumor areas as negative. For L368, laboratories 2, 3 and 4 had positive staining in normal ducts. Laboratory 1 reported weak staining in normal ducts with L368. Within tumor areas, all four laboratories indicated loss of staining for L368 with heterogeneity of staining in more well-differentiated areas of the tumor. While there was complete concordance for lack of staining with LGII, Laboratory 1 noted some staining within the luminal epithelium of the normal ducts.

In case 3, there was concordance for staining of the tumor areas with all 3 antibodies among the four centers. Tumor areas for HC10, L368 and LGII were negative in undifferentiated

areas of the tumor, with heterogeneous staining in the well- differentiated areas (25%) for HC10 and L368. Normal breast epithelium was scored as weakly staining by Laboratory 1. Normal breast epithelium was not present in the tissue sections examined by Laboratory 2, 3, and 4. Stromal cells were present and scored as positive among all four laboratories.

The quality of the staining and the overall results were similar despite variations to the protocol. Microwave and citrate buffer antigen-retrieval substitutions yielded comparable results. Laboratory 2 demonstrated that a negative control is useful as an internal standard for reagent optimization and for noting any non-specific reactivity. CD45 staining as employed in Laboratory 1 may be helpful in identifying the infiltrating lymphocytes in germinal centers and dendritic cells as seen in case 1.

Microsatellite Analysis

Lab 1, Lab 2 and Lab 4 performed Microsatellite analysis. Loss of heterozygosity was observed at the β2 microglobulin locus in Case 1 and 3, concordant with down regulation at the level of HLA expression. This locus was either normal or uninformative in Case 2. For the HLA class I loci at 6p21, LOH for three 6p21 markers suggested concordance with down regulation of class I antibodies, HC-10 and L3682 in case 1. For case 2, 6p21markers were either uninformative, normal or did not yield an interpretable result. For case 3, 6p21 markers indicated LOH for D6S105 and microsatellite instability (MSI) for maker marker D6S265 by Lab 4, as compared to normal and uninformative results, respectively, by Lab 1 (Table 3).

For an interpretation of LOH, there was concordance, except for marker D6S105 in Case 1, which was scored as LOH by Labs 1 and 4, and homozygous by Lab 2. In case 3, LOH for D6S105 and MSI for maker marker D6S265 by Lab 4 were scored as normal and uninformative respectively, by Lab 1. Among laboratories, for case 1, all three indicated LOH for markers D6S265 and D6S276 (Figure 1), and LOH for D15S209 by Lab 1 and Lab 4. For all three cases, lab 1 and Lab 4 concordantly interpreted marker D6S1618 as homozygous (uninformative). Lab 1 and 4 noted homozygosity for marker D15S126 in case 1; Lab 2 did not generate an interpretable result.

Discrepant results were noted in Case 2, for marker DS105; Lab 1 indicated heterozygozity in the normal and tumor, whereas Lab 2 and Lab 4 indicated a homozygous result. This was also the case for marker D15S209 in case 2, which was scored as heterozygous (normal) by Lab 1 and homozygous (uninformative) by lab 4. In case 3, LOH was indicated by Lab 4 for marker D6S105, and microsatellite instability (gain of an allele as compared to a homozygous status in the normal) for D6S276, an interpretation that was not shared by Lab 1.

Discussion

Immunohistochemical (IHC) staining of frozen sections of malignant tumors has shown that abnormalities in HLA class I antigen expression are frequent in malignant cells ((1-5). Despite the possible clinical significance and potential for T cell-based immuno-therapy (2), evaluation of malignant lesions for HLA class I antigen expression is not performed routinely, even for patients who are candidates for such therapy. This reflects, at least in part, reluctance by pathologists to utilize frozen tissue sections in IHC assays.

The potential predictive value of HLA expression in evaluation and therapy planning has been under-utilized. The importance of this marker in the evaluation of breast cancer patients prompted our undertaking of a multicenter study approach to develop and standardize an IHC

protocol using formalin-fixed, paraffin-embedded tissue and anti-HLA mAbs. Anti-HLA HC-10, a mAb to a determinant expressed on β2-microglobiulin free HLA class I heavy chains (chromosome 6p21), LGII, anti-HLA class II mAb (chromosome 6p21), and L368, an anti-human β2- microglobulin (chromosome 15q21) were evaluated in sections of archival formalin-fixed paraffin embedded breast tumors. Positive staining with CD45 was useful in accounting for lymphocyte and dendritic cell populations in tumor areas that were otherwise predominantly negative with all three HLA antibodies.

HLA class I antigen downregulation in conjunction with and without heterogeneity was concordantly scored in tumor regions of all cases with HC10 and mAb L368. Furthermore, no staining of either normal or malignant breast tissue was detected by the four laboratories in lesions stained with LGII, however, lymphocytes as expected were strongly stained in the same section. While there was some lack of agreement with staining interpretations in normal breast epithelium for L368, lymphocytes and stromal cells were judged positively stained by all four laboratories.

DNA analysis from archival tissue permits retrospective characterization of disease and has facilitated molecular data gathering from large population-based epidemiological study cohorts. It can also serve as surrogate tissue material for genetic testing of diseases or syndromes in the absence of fresh or frozen tissue or peripheral blood lymphocytes. In retrospective studies for DNA marker characteristics, tissue is mostly available in the form of formalin-fixed paraffin tissue blocks. For the detection of allelic imbalance in tumor DNA, paired normal and corresponding tumor tissue is necessary. Lack of or scarcity of normal or tumor tissue in the paraffin sections or poor quality of the DNA due to the use of inadequately buffered formaldehyde during the fixation process presents serious limitations to studies that rely on archival patient tissue resources.

Discordant results and lack of interpretable results in some instances reflect in part low DNA yields and poor quality DNA that become factors in interpretation of low signal allele peaks, stutter peaks and difficulty with amplification of larger size alleles due to degradation of DNA inherent in formalin-fixed archival tissue resources. For this study, limited resources that required a four-way distribution hampered availability of DNA for repeat sampling in some cases. Despite these challenges, for markers D6S276 and D6265, an interpretation of LOH was validated by all three participating centers, and D15S209 by at least two laboratories.

Conclusion

The results indicate that formalin fixed paraffin embedded tissue represent a useful substrate to monitor HLA antigen expression in malignant lesions, especially when appropriate markers are also used to differentiate malignant cells from lymphocytes and dendritic cells. Loss of heterozygosity for markers representative of the HLA loci at 6p21 and 15q21 agreed with down regulation of expression of class 1 gene expression. LOH associated for loss of the HLA locus at chromosome 6p supports an extended mechanism that may contribute to HLA haplotype loss previously described in different histological tumor types (9-11). Contaminating stroma can mask LOH results and microscopic or laser microdissection to separate stroma and tumor would facilitate an interpretation of LOH in cases with unclear results.

Table 1 Panel of Microsatellite Markers

Marker	location	size	alleles	%het	sequence
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D6S-1618	6p distal (TAP-1)	132-164	14	0.86	5′GGCCTGAGCAGTGCAT3′ 5′TGATTCCTAATCTGCGGG3′
D6S-291	6p21.3-6p21.2 Class II, TAP-2	198-210	7	0.73	5′CTCAGAGGATGCCATGTCTAAAATA3′ 5′GGGGATGACGAATTATTCACTAACT3′
D6S-273	6p22.3-6p21.3 Class III, HLA-B	120-140	8	0.77	5′GCAACTTTTCTGTCAATCCA3′ 5′ACCAAACTTCAAATTTTCGG3′
D6S-265	6p22.3-6p21.3 Class I, HLA-C	122-138	12	0.78	5′ACGTTCGTACCCATTAACCT3′ 5′ATCGAGGTAAACAGCAGAAA3′
D6S-105	6p22.I-6p21.33 Class I, HLA-A	116-138	12	0.79	5′GCCCTATAAAATCCTAATTAAC3′ 5′GAAGGAGAATTGTAATTCCG3′
D6S-276	6p22.3-6p21.3 Class I	198-230	14	0.83	5′TCAATCAAATCATCCCCAGAAG3′ 5′GGGTGCAACTTGTTCCTCCT3′
D15S-126	15q21 (β2-microglobulin gene)	188-218	11	0.82	5′GTGAGCCAAGATGGCACTAC3′ 5′GCCAGCAATAATGGGAAGTT3′
D15S-209	15pter-15qter	189	---	0.78	5′AAACATAGTGCTCTGGAGGC3′ 5′GGGCTAACAACACTGTCTGC3′

% het.= % heterozygosity

Table 2 **Summary of IHC Validation Results**

Results	Case 1								
Antibody	HC-10		Outcome	L368		Outcome	LGII		Outcome
	Normal	Tumor		Normal	Tumor		Normal	Tumor	
Lab 1	‡	‡	loss	‡	‡	loss	‡	‡	normal
Lab 2	‡	‡	loss	‡	‡	loss	‡	‡	normal
Lab 3	‡	‡	loss	†*	‡	loss	‡	‡	normal
Lab 4	‡	‡	loss	†	‡	loss	‡	‡	normal

Results	Case 2								
Antibody	HC-10		Outcome	L368		Outcome	LGII		Outcome
	Normal	Tumor		Normal	Tumor		Normal	Tumor	
Lab 1	‡	‡	loss	†*	‡	loss	‡*	‡	normal
Lab 2	‡	‡	loss	‡	‡	loss	‡	‡	normal
Lab 3	‡	‡	loss	‡	‡	loss	‡	‡	normal
Lab 4	†*	‡	loss	‡	‡	loss	‡	‡	normal

Results	Case 3								
Antibody	HC-10		Outcome	L368		Outcome	LGII		Outcome
	Normal	Tumor		Normal	Tumor		Normal	Tumor	
Lab 1	‡	‡	loss	†*	‡	loss	‡	‡	normal
Lab 2	‡	‡	loss	‡**	‡	loss	‡	‡	normal
Lab 3	‡	‡	loss	‡**	‡	loss	‡	‡	normal
Lab 4	‡	‡	loss	‡**	‡	loss	‡	‡	normal

‡ = complete concordance
† = incomplete concordance
‡* = complete concordance, see text
†* = incomplete concordance, see text
‡** = stromal cells and lymphocytes

Table 3 : Loss of Heterozygosity Results

Lab 1=Garrido, Lab2 = Tilanus, Lab 4= Worsham

Markers	Case 1						Case 2						Case 3					
	Lab 1		Lab 2		Lab 4		Lab 1		Lab 2		Lab 4		Lab 1		Lab 2		Lab 4	
	N	T	N	T	N	T	N	T	N	T	N	T	N	T	N	T	N	T
D6S1618	Nr	h	nd	nd	h	h	**h**	**H**	nr	nr	**h**	**h**	**h**	**h**	nr	nr	**h**	**h**
D6S291	Nr	ht	nd	nd	nr	ht	nr	Nr	nr	nr	nr	h	nr	nr	nr	nr	nr	?ht
D6S273	**Ht**	**loh**	nr	nr	**ht**	**loh**	h	H	nr	nr	nr	h	ht	ht	nr	nr	nr	ht
D6S265	**Ht**	**loh**	**ht**	**loh**	**Ht**	**loh**	ht	Ht	nr	nr	nr	?ht	ht	ht	nr	nr	nr	ht
D6S105	**Ht**	**loh**	***h***	***h***	**ht**	**loh**	***ht***	***Ht***	***h***	***h***	***h***	***h***	***ht***	***ht***	nr	nr	***ht***	***loh***
D6S276	**Ht**	**loh**	**ht**	**loh**	**ht**	**loh**	**h**	**H**	nr	nr	**h**	**h**	***h***	***h***	nr	nr	***h***	***ht***
D6S311	**H**	**h**	nr	nr	**h**	**h**	nr	Nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr
D15S126	**H**	**h**	nr	nr	**h**	**h**	nr	Nr	nr	nr	h	h	nr	nr	nr	nr	ht	loh
D15S209	**Ht**	**loh**	nr	nr	**Ht**	**loh**	***ht***	***Ht***	nr	nr	***h***	***h***	ht	nr	nr	nr	ht	loh

N= normal; T= tumor; nr= no result; nd= not done; h= homozygosity; ht=heterozygosity; LOH= loss of heterozygosity
bold, non-italicized=concordance; bold, italicized=lack of concordance.

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FIGURE LEGENDS

Figure 1: Loss of heretozygosity in Case 1 with marker D6S276. The A2 allele is significantly reduced in the tumor as compared to the normal

Figure 2: Normal breast epithelium in Case 1 (Figure 2A) and corresponding tumor (Figure 2B), low power (10X) representations on the left side, high power (40X) on the right.

Figure 2A: Normal breast epithelium (Case 1) is uniformly stained with HC-10 and with some degree of heterogeneity with L368 (B2m). Note complete lack of staining with class II LGII mAb and with CD45 in normal breast epithelia areas, with intense staining of infiltrating lymphocytes.
Figure 2B: Tumor epithelium (Case 1) indicates heterogeneous staining with class I mAb HC-10. Note complete loss of B2m, LGII and CD45 in tumor areas, with intense staining of infiltrating lymphocytes and dendrite cells (CD45).

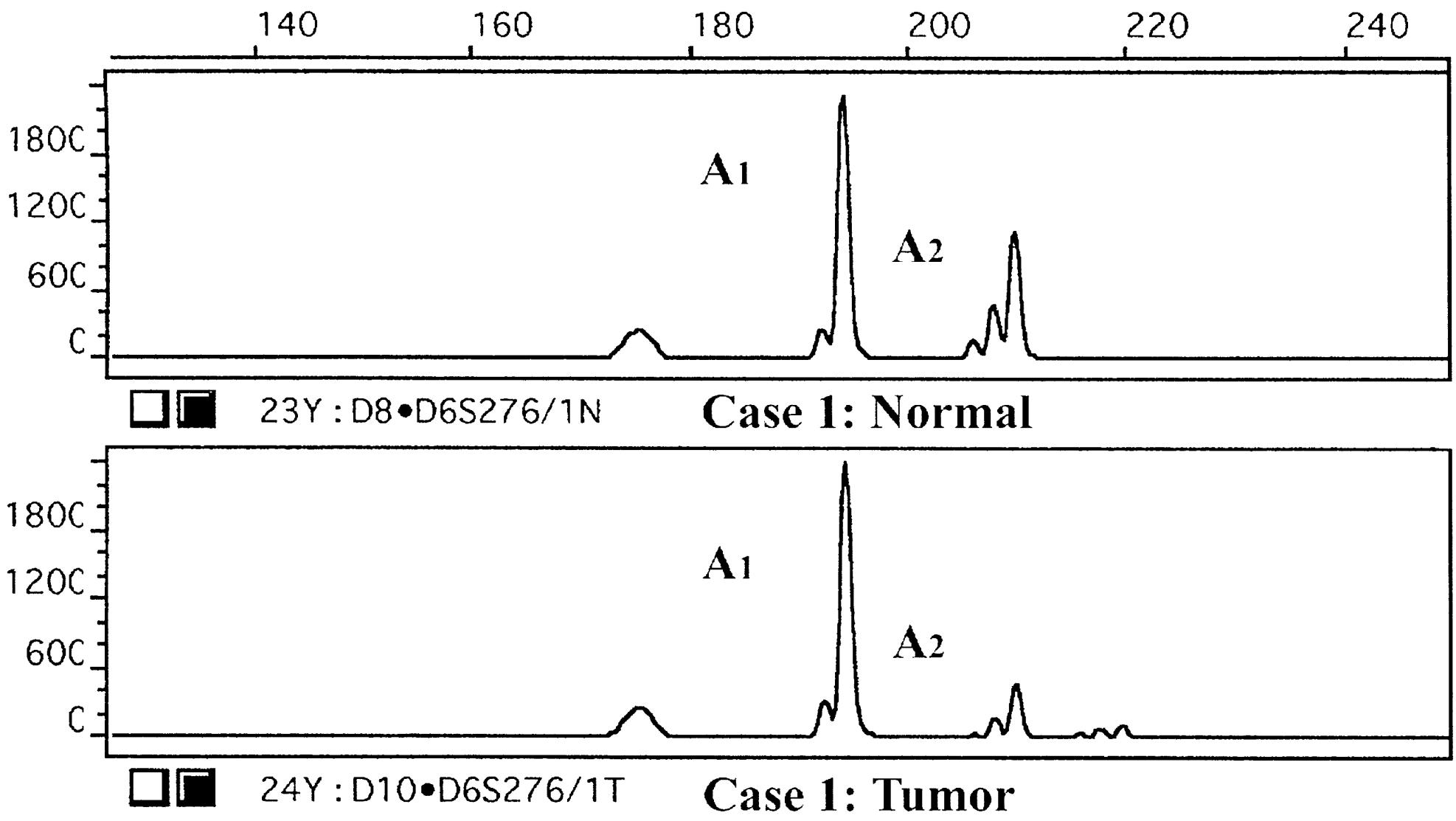

140
160
180
200
220
240
180C
120C
60C
C
A1
A2
23Y : D8•D6S276/1N
Case 1: Normal
180C
120C
60C
C
A1
A2
24Y : D10•D6S276/1T
Case 1: Tumor

Figure 2A

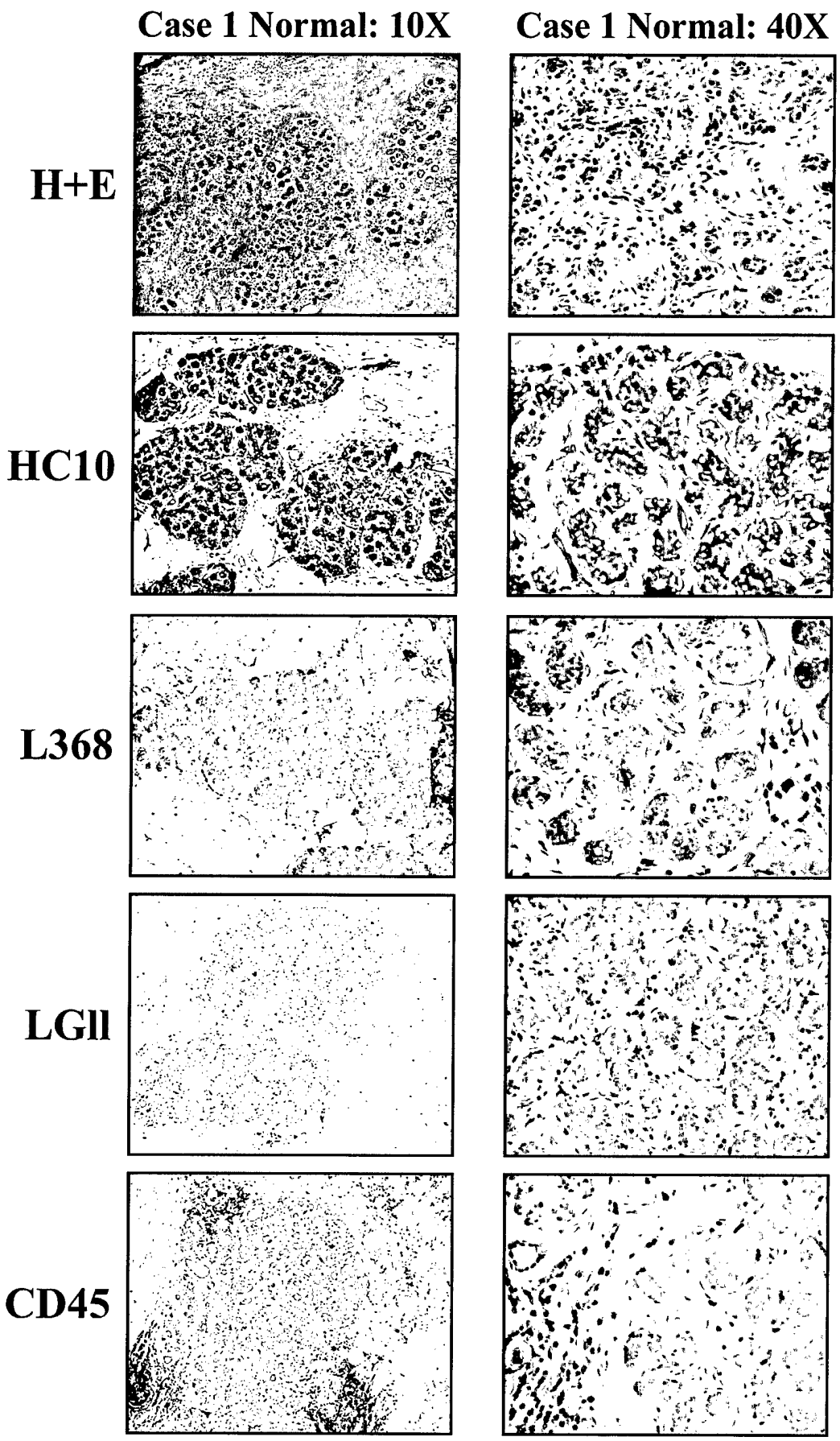
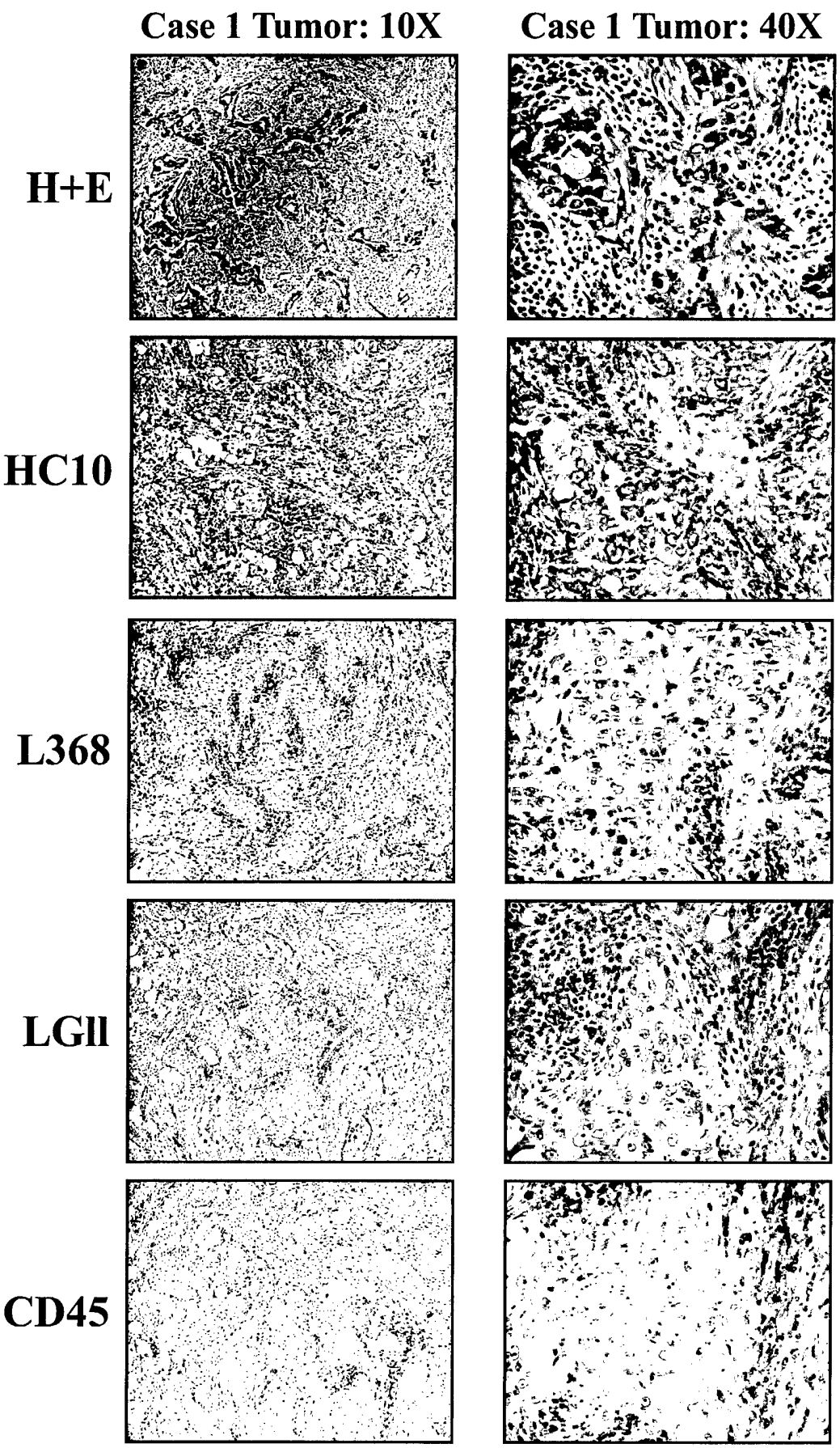


Figure 2B



Loss of heterozygosity at HLA loci 6p21 and 15q21

Maria J. Worsham

Distinct mechanisms have been found to underlie the multiple tumor HLA class I immuno-surveillance escape phenotypes which have been identified by immunohistochemical staining of frozen tissue sections with mAbs which recognize monomorphic, locus-specific and allele-specific determinants of HLA class I antigens. HLA haplotype loss in tumor cells has been shown to be generated frequently by total or partial deletion of chromosome 6, which carries the major histocompatibility complex in humans (1-3). The detection of these losses has been facilitated by the recent identification of highly polymorphic microsatellite markers (short tandem repeats). Utilizing short tandem repeats mapped on chromosome 6 (6p21, HLA locus) and on chromosome 15 (5 q21 locus, β2 microglobulin), loss of heterozygosity differences have been described in the frequency of HLA class I genes and of β2m genes in various types of malignancies. The values range from about 15% in colorectal carcinoma lesions to about 50% in head and neck squamous cell carcinoma (1, 2) and in cervical carcinoma lesions (3). Whether these differences reflect the use of different markers, lack of standardization in the methodology used, and/or different characteristics of the malignancies and/or patients investigated remains to be determined.

To determine if loss of expression of MHC Class I molecules at the protein level reflect alterations at the gene level, the HLA and Cancer component of the 13th International Histocompatibility Workshop undertook a small pilot multicenter study. The goal was to optimize and assess the reproducibility of the assays to measure the expression of HLA class I antigens and to detect LOH at the HLA locus (6p21) and at the β2m locus in formalin fixed, paraffin embedded normal breast epithelium and malignant breast lesions.

DNA from microdissected normal and tumor tissue were evaluated with microsatellites at the MHC class I 6p21.3 locus (HLA-A, B, C determinants) and at the 15q21 beta 2 microglobulin locus for concordance of expression (Table 1). HLA class I antigen down-regulation in conjunction with cellular heterogeneity of expression in three breast carcinoma cases was concordantly reported by the four participating laboratories with the anti-HLA class I antibody HC-10 and with the anti-beta 2 microglobulin L368. Furthermore, the four laboratories detected no staining of normal or malignant breast tissue for the lesions stained with the anti-HLA class II LGII. In contrast, infiltrating lymphocytes were strongly stained by LGII. Downregulation of class 1 was reflected by LOH in cases 1 and 3 for the 15q21 locus and in case 1 at the 6p21 locus. The results indicate that FFPTs represent a useful substrate to monitor HLA antigen expression in malignant lesions, especially when appropriate markers are used to differentiate malignant cells from lymphocytes and dendritic cells.

Loss of heterozygosity for markers representative of the HLA loci at 6p21 and 15q21 agreed with down regulation of expression of class 1 gene expression. LOH associated with loss of the HLA locus at chromosome 6p supports an extended mechanism that may contribute to HLA haplotype loss previously described in different histological tumor types (1-3). Contaminating stroma can mask LOH results and

microscopic or laser microdissection, to separate stroma and tumor would facilitate an interpretation of LOH in cases with unclear results.
(Worsham et al. manuscript submitted-ref 4).

Molecular methods: loss of heterozygosity

Processing Tumor Specimens for DNA Analysis

DNA from normal and tumor tissue was extracted from 4-5 micron sections from archival paraffin embedded tissue blocks. DNA was extracted using the QIAamp Kit (Qiagen Inc. Chatsworth, CA) according to the manufacturer's protocol.

Microsatellite analysis

A minimal microsatellite panel to detect LOH at the HLA locus at 6p and the β_2m locus at 15q included D6S1618, D6S291, D6S273, D6S265 and D6S311, and D15S126 and D15S209, respectively (Table 1). Fluorescent-labeled primers were obtained from Perkin-Elmer Applied Biosystems (Foster City, CA). 2µl-4µ of purified paraffin DNA from a total elution volume of 200ul from normal paraffin tissue and tumor in each case was used for each PCR reaction. PCR was performed in a 1X PCR buffer (10mM Tris-HCL pH 8.3, 50 mM KCl), 2.5mM $MgCl_2$, 200 uM each dNTPs, 1uM of each primer and 0.8 units of Ampli Taq Gold polymerase (Perkin-Elmer) in a 10ul reaction volume, using a Perkin Elmer GeneAmp 9600 followed by 35 cycles, 12 min 95 °C initial denaturation, an additional 50sec denaturation at 94 °C, 60 sec annealing at 57 °C and 60 sec extension at 70 °C. Amplification was completed with a final incubation step at 72 °C for 30 minutes.

The amplified PCR products were analyzed using an automated ABI PRISM sequencer (model 310, PE Applied Biosystems), using GeneScan Rox 400 size standard (PE Applied Biosystems). In brief, 12 ul deionized formamide were combined with 0.5 ul GeneScan Rox 400 size standard (PE Applied Biosystems) and 2ul of PCR product in a Genetic Analyzer sample tube. The tubes were closed with Genetic Analyzer septa and, after short mixing (vortex), the samples were denatured in a heat block for 4 minute at 90 °C, chilled on the ice, and spun briefly in a microcentrifuge in order to collect the contents. The samples were loaded on the ABI 310 genetic Analyzer and the run in accordance with the manufacturer's protocol (5).

Interpretation Criteria

A sample was defined as heterozygous if there were two distinct alleles for a given marker, i.e., maternal and paternal alleles were distinguishable (informative for LOH). The presence of only one size allele for a given marker, i.e., maternal and paternal alleles are indistinguishable (uninformative for LOH) was interpreted as homozygous. Loss of one of the two constitutive alleles in the tumor was scored as loss of heterozygosity (LOH). LOH was assigned when the fluorescent signal of one of two alleles was reduced by more than 30% as compared with the heterozygous control normal DNA sample .

ACKNOWLEDGEMENTS: Supported by the Department of Defense DAMD17-00-1-0288 and NIH R24 CA84497

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